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INTERNATIONAL SYMPOSIUM ON THE CELLULAR BASIS OF DIFFERENTIATION

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Some Introductory Remarks on the Cellular Basis of Differentiation

by PAUL WEISS[1]

University of Chicago

I. INTRODUCTION

BY request of the committee which organized the conference on 'The Cellular Basis of Differentiation', I undertook to open the meeting with a sort of topical outline that might serve as a guide in the subsequent free and informal discussion. In view of the diverse meanings attached to the term 'differentiation' in various quarters, from development in general in the broadest sense to the production of visible specialized cell structures in the narrowest sense, it seemed desirable to circumscribe at least the scope within which the conference was to hold itself. The instructions of the organizing group were to focus the discussion on the cellular transformations which lead to the final functional state of the mature cell. This limitation is taken into account in the following outline. At the same time it is clear that terminal cell specialization is part of a continuous process, hence cannot be neatly separated from antecedent developmental processes leading up to it. Therefore the difference between this presentation and others dealing with early embryonic phases lies in the emphasis rather than in the nature of the objects. In fact, it is becoming ever more evident that the study of special histogenesis and organogenesis may cast as much light on the interpretation of early embryonic processes as the study of early events, e.g. the formation-of the fertilization membrane or of the sperm tail, contributes to the understanding of specific terminal cytodifferentiation. Accordingly, the main objective of the following outline is to set the problem of cellular differentiation into proper focus and perspective within the broad problems of ontogeny.

The term 'differentiation' has long been used vaguely and loosely; longer than is profitable for either the description and interpretation of facts or the formulation of problems or the design of experiments. The results of a conference such as this should be gauged by its success in reducing this vagueness and looseness.

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Since science benefits not only from the discovery of new facts but also from the elimination of inconsistencies and misconceptions caused by unfamiliarity with, or disregard of, known facts, a first prerequisite seemed to be for this conference to have before it a complete inventory, as it were, of all the many facts and facets normally subsumed under the single term of 'differentiation'. Such a table of contents of the problem was therefore prepared and distributed to the members of the conference in advance of the meeting. It is reproduced below. A second task that seemed called for was the following.

As on past occasions (1947, 1949, 1950) to which the reader may be referred for further details, I found it necessary in order to deal profitably with the problem of cellular differentiation, to operate with a more realistic and workable conception of 'cell' and 'protoplasm' than is normally implied in these symbolic terms. I have sketched this conception previously under the name of 'molecular ecology'. For brevity and pregnancy, I shall condense some of its major aspects into diagrams presented here for the first time. They are admittedly greatly oversimplified models, intended merely to help us to visualize the phenomena and to formulate problems more cogently. They permit us to phrase specific questions which can be answered decisively. As the answers come in, those models will be validated or invalidated or, most likely, appropriately modified. In the meantime they will have served as a temporary scaffolding for the erection of a more tangible and less non-committal notion about 'differentiation' than we now possess. Therefore no more than this tentative pragmatic value need be attached to them.

Differentiation is not something that should or could be defined, at least not in our present state of ignorance and conflicting opinions. It is a summary term for a great many diverse experiences and evidently its content will grow and change as more experiences are gathered. Hence whatever definition would be attempted would have no finality. To strip the term of its vagueness would not require definition so much as circumscription, and this is what will be attempted in the following. Instead of engaging in the illusory task of defining the properties of some non-existing entity, called 'differentiation', we shall simply list those properties of differentiating living systems that have been observed. This list is a mere sample with no claim to being either categorical or exhaustive. It tries to break down the general problem into a number of specific issues which can serve to bring some preliminary order into the enormous mass of scattered data bearing on the topic.

A Programmatic Inventory of Cytodifferentiation

A. INTRODUCTION TO THE PROBLEM OF DIFFERENTIATION

Progressive changes in cell *strains* versus the terminal specialization of cell *individuals*. Changes in composition versus changes in distribution and arrangement. Which parts of the cellular system (*a*) change with time in all cells equally? (*b*) change differentially according to cell type? (*c*) remain unchanged throughout differentiation?

B. Criteria of cytodifferentiation

(By what signs can we recognize differentiation?)

1. *Morphological criteria* (What signs of differentiation are either immediately visible or can be rendered visible by appropriate tests?)
   - (*a*) Microscopic characters (intracellular and intercellular structures; their sizes, aggregations, localizations and modifications).
   - (*b*) Submicroscopic characters (polarization- and electron-optical identification; elemental particulates; orientation; patterned versus irregular deposition; relation of artifacts to reality; microstructural basis of cell shape).
   - (*c*) Microchemical organization (differential staining reactions, including electron stains; formed secretions; enzyme localization; pH patterns; &c.).

2. *Direct physiological criteria* (What signs of differentiation are revealed by changes in the physiological properties of cells and their discharges?)
   - (*a*) Functional activities (metabolic activity; secretions; phagocytosis; contractility; selective absorption and storage, as of iodine by the thyroid cell).
   - (*b*) Changes in composition (chromatography; ionic composition; changes of identifiable macromolecular compounds, including proteins, enzymes, antigens, nucleic acids, &c.).
   - (*c*) Changes in chemical composition and distribution determined by ultraviolet and infra-red absorption.

3. *Indirect physiological criteria* (What signs of differentiation not directly observable can be deduced from differential reactions of the living cell?)
   - (*a*) Selective reaction to drugs, hormones, viruses, &c.
   - (*b*) Organ-specific serological interactions.
   - (*c*) Self-sorting of cells according to type (e.g. following dissociation; in wound healing; &c.).

C. Mechanisms and causative agents of cytodifferentiation

(The detailed story of just where and how the various criteria of differentiation arise, and of the intracellular and extracellular factors and conditions upon which they depend).

1. *Origin of specialized cell products*
   - (*a*) Sites of specific synthesis and conversion of protoplasmic systems (role of nucleus, genes, cytoplasmic granules, mitochondria, centrosomes, &c., in the manufacture of specialized cell products; continuity of microscopic and submicroscopic 'self-duplicatory' bodies).
   - (*b*) Secondary rearrangements (coacervation; polymerization; fibril and membrane formation; formation of cilia, sperm tails, insect hairs and scales, keratinization, &c.).
   - (*c*) Extrusion of cell products (formation of intercellular cements, ground substances, myelinization, chitinization, &c.).
   - (*d*) Regeneration of cell parts (restoration of intracellular structures in functional cycles and after injury, including merocrine gland cells, protozoans, neurons, muscle-fibres, &c.; fate of differentiated structures during and after cell division).

2. *Metabolism of cytodifferentiation* (What are the general metabolic requirements for the functional and structural specialization of the cell?)

   (*a*) Thermodynamic considerations and energy requirements for differentiation (relating the metabolic patterns in maintenance, growth, differentiation, and functional operation of the cell).
   (*b*) Comparative aspects (relating the metabolic requirements of different cell types to their specialized features, such as cell size, cell life-span, nucleo-plasma ratio, elongation, cell shape, &c.).

3. *Specific accessories to cytodifferentiation* (What specific contributions and aids from the environment are prerequisite for the expression of given cytodifferentiations?)

   (*a*) Specific nutritive accessories (vitamin A in retinal differentiation; ascorbic acid in connective tissue differentiation; pterins in insect pigments).
   (*b*) Hormones (general hormonal requirements for cytodifferentiation; specific hormone-dependence of hormonal end-organs; differentiative interdependence of endocrine glands; neural influences on differentiation).
   (*c*) Biochemical genetics of defective cytodifferentiation (e.g. albinism, chondrodystrophia, sickle-cell disease, &c.).
   (*d*) Cellular interactions in differentiation (effects of organ-specific discharges other than hormones; contact influences transmitted from cell to cell; homoeogenetic inductions; 'infective' propagation of differentiation, &c).
   (*e*) Physical factors (orienting and aligning forces in the cellular environment in their relations to cell polarization, locomotion, and structure formation; pressure and tension as factors in muscular, skeletal, and connective tissue differentiation; diffusion and hydrodynamic factors in differentiation; the role of interfaces, &c.).

4. *Pluripotency of differentiation* (What is the basis for the fact that most cells prior to their terminal stages are capable of several alternative courses of cytodifferentiation?)

   (*a*) Multivalency of cellular equipment (all-or-none principle of differentiation; physical basis of 'threshold', 'dominance', and 'pacemaker' functions, &c.).
   (*b*) 'Position' effects producing cellular dimorphism (e.g. terminal secretory versus duct cells in glands; endothelial versus blood cells; egg versus nurse cells; ganglion versus satellite cells, &c.).
   (*c*) Proliferation and differentiation (antagonistic relations between reproduction and specialization; occurrence and significance of differential cell divisions; endomitosis in relation to cytodifferentiation, &c.).

D. GENERAL PROBLEMS AND CONCLUSIONS

1. *'Dedifferentiation' and 'redifferentiation'* (To what extent does the loss of certain criteria of differentiation signify (*a*) loss of type specificity, and (*b*) true reversion to pluripotent condition, implying the capacity to differentiate in a variety of new directions?)

2. *Genes, nucleus, and cytoplasm in differentiation* (it is essential to proceed beyond the trivial assertion that they all are involved in the process of differentiation, and to

examine just how and when each component participates; including the hypothetic role of 'plasmagenes', somatic mutations, polyploidy, &c.).

3. *Comparative aspects* (What lessons regarding cytodifferentiation can be gained from the study of differentiation in bacteria, slime moulds, protozoans, fungi, and higher plants?)

4. *Cytodifferentiation and morphogenesis* (How cytodifferentiation determines tissue and organ formation).

5. *Cytodifferentiation and carcinogenesis* (Is the primary deviation of the cancer cell a matter of aberrant growth or rather of defective cytodifferentiation? If the latter, which ones among the multiple features of the complex differentiation process enumerated above are predominantly involved?)

With this index as a background, we shall now proceed to single out a number of specific points for discussion.

II. CONNOTATIONS OF 'CYTODIFFERENTIATION'

Ordinarily the term 'cytodifferentiation' is used interchangeably in at least four different meanings. It designates:

1. The variety of terminal characters distinguishing the cells of a developed organism; see, for instance, the frequent statements to the effect that 'a given organism shows such-and-such differentiations'.
2. The process by which a cell of more generalized microscopic appearance assumes the more specialized aspect referred to in point 1 by elaborating distinct 'differentiation products' characteristic of its kind, declaring colour, as it were; e.g. the differentiation of myofibrils in a muscle cell, of melanin granules in a pigment cell, of hemoglobin in an erythrocyte, of colloid in a thyroid cell, of neurofibrils and Nissl bodies in a nerve cell, of keratin in an epidermal cell, &c.
3. The process by which the cells expressing themselves visibly in this fashion develop the basic equipment to do so; e.g. the differentiation (or 'maturation') of a myoblast into a muscle cell; of a melanoblast into a chromatophore; of a hemocytoblast into a blood cell; of a thyroblast into a thyroid cell; of a neuroblast into a neuron. Evidently point 3 grades into point 2, and the empirical distinction between them merely recognizes the fact that even the 'mature' muscle-fibre can still form new myofibrils (as in hypertrophy), the 'mature' pigment cell can still produce pigment, and so forth.
4. The process by which each cell type labelled here as '-blast' acquires the peculiar physico-chemical machinery that enables it to manufacture the specialized protoplasm referred to under point 3, and in many cases not in just one single set, but in an unlimited number of identical sets, as myoblasts proliferate myoblasts, melanoblasts more melanoblasts, and so forth. Whether the proliferating cells are strictly localized ('germinal cords' or 'germinal layers', e.g. in the vertebrate skin, the blood-forming centres, the intestinal mucosa), or whether

they are widely scattered, the basic question remains of how they have come by their differential faculties of reproducing each a characteristic type of protoplasmic descendants identified by the specific performances listed (chronologically reversed) in points 1 to 3.

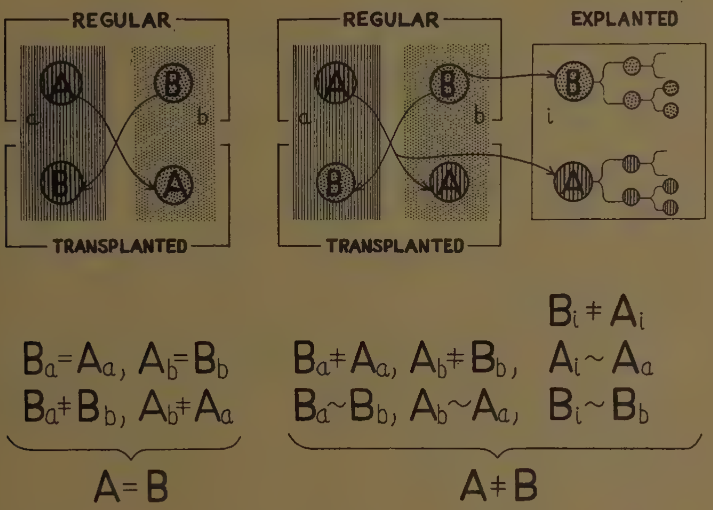
Point 4 thus raises the question of the origin of divergencies among initially identical cell strains. From the testimony of Experimental Embryology and Pathology we know that the same cell can be the progenitor of a variety of derivative types—the more the earlier we test it—hence diversification among descendants of common precursor cells is a fact. The problem of how this diversity comes about is of a different order than that of the subsequent steps 3, 2, and 1, which merely elaborate an already existing diversity. Even so, since the phenomena 4, 3, 2, and 1 are continuous in time, it seems valid for purposes of our discussion, and probably quite generally, to identify differentiation with the whole series of physico-chemical changes described by these four points. Consequently we can state that differentiation (*a*) is a complex (as opposed to unitary) phenomenon; (*b*) is a stepwise process, not an abrupt event; and (*c*) can be meaningfully referred to only in terms of the stage which that series of processes has reached at a given time. To call a cell either 'undifferentiated' or 'differentiated' without further specification is not only inaccurate but scientifically meaningless.

Ideally one would have to follow a cell and its descendants through development, sample it at various stages along this course, and describe its properties objectively at each sampled stage: the changes of properties registered over the whole course would then add up to a complete record of 'differentiation'. Regardless of whether or not such objective description is ever attainable, we can at least come closer to it by including in our description as many properties as we can possibly identify, instead of limiting ourselves arbitrarily to those few properties which happen to reveal themselves optically or in some other overt manner. Any test, however indirect, and any reaction, however much delayed, that helps us to distinguish between two cells (or the same cell at different times) is a pertinent index of distinctive cellular 'properties'. The above Inventory of Cytodifferentiation illustrates the great variety of covert signs of differential cellular constitution that could properly be used for this purpose. The study of differentiation thus resolves itself into (*a*) the identification of criteria of the state of a cell; (*b*) the comparison of these criteria, revealing systematic differences between them; and (*c*) the exploration of the origin of these differences as they arise between (α) a given cell at an earlier stage and the same cell at a later stage, (β) a given cell and its descendant cells, and (γ) different descendant lines stemming from equivalent (or 'equipotent') precursor cells.

Differentiation thus connotes the appearance of *true* differences of constitution —'progressive transformation'—within a protoplasmic continuum extending along the time line, regardless of whether or not this continuum undergoes further subdivision in space by successive cell divisions or remains a continuous mass. *Identical* courses from identical starts can produce differences only *within*, but not between, protoplasmic strains. The emergence of true differences *between* strains derived from identical sources is due to the fact that their transformations have taken *divergent* courses.

III. TESTS OF DIFFERENTIATION

The term 'true differences' implies a distinction between characters inherent in the cell and features reflecting its environment. Now, evidently a cell without an environment is a fiction; hence no property or manifestation of a cell can be divorced from a consideration of the environment in interaction with which it has occurred or been displayed. However, by comparing the same cells in different environments, and different cells in the same environment, their relative contributions to a given reaction, transformation, and eventually form, can be assessed. These behavioural tests of cell *character*, in contradistinction to environmental expression, are illustrated in the diagram, Fig. 1. It shows in the left third, two cells, *A* and *B*, in their regular environments, *a* and *b*; the conformance between cell and environment being indicated by identical markings, that is, stripes for *a* and dotting for *b*. Cell *A* is then transplanted into environment *b*, and cell *B* reciprocally into environment *a*. The same operation is carried out in the middle diagram. Yet the subsequent behaviour of the transplanted cells is radically different in the two diagrams. In the left one the transposed cells *A* and *B* conform entirely to their new environments, proving that the erstwhile distinctions had not resided in any ingrained differences between the two cells but had been expressions of different behaviour of a single kind of cell in different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$B_a = A_a,\ A_b = B_b$
$B_a \neq B_b,\ A_b \neq A_a$
$A = B$
$B_i \neq A_i$
$B_a \neq A_a,\ A_b \neq B_b,\ A_i \sim A_a$
$B_a \sim B_b,\ A_b \sim A_a,\ B_i \sim B_b$
$A \neq B$

FIG. 1

environments. In the middle diagram, by contrast, the cells after transposition continue to show properties referable to their origin and do not adopt each other's former properties and appearances. Such behaviour is incontestable proof that some intrinsic differences had existed between *A* and *B* prior to their transfer. This does not exclude, of course, that these differences might have been conferred upon them by their previous prolonged residence in those environments. Of this we shall speak later. Nor does this test imply that the transposed cell would not show some response to the new environment. In most cases it will, but the salient feature remains that *A* in *b* does not become like *B* in *b*, and *B* in *a* does not become the like of *A* in *a*.

It can readily be seen that this scheme is at the base of most classical experiments on embryonic transplantation, in which a change of behaviour from that of the left to that of the middle diagram has been ascribed to a process of 'determination'. In the conviction that determination is based on real physico-chemical changes, and since on the other hand we have extended the term of differentiation to include all indices of transformation, and not just the accidentally visible ones, there seems to be no further justification for retaining these two separate categories on the cellular level. Determination then is but the earlier part of the differentiation process, which is less directly discernible. But power of discernment is a property of the observer and his tools, and not of the observed system.

It is evident that these diagrams of behavioural tests apply equally well to the relation between genotype and phenotype. If *A* and *B* are taken to be eggs (or seeds) raised in media (or soils) of different compositions, the left diagram evidently represents phenotypic variation, while the middle one reveals genotypic differences. It is important, however, to bear in mind that inherent differences, which in the zygote state would ordinarily indicate differences of genotype, are manifested among somatic cell strains despite their supposedly identical genic equipment (see below).

This latter fact is best demonstrated by the most cogent test of differential constitution, namely, that diagrammed in the right-hand panel of Fig. 1, representing transfer of cells *A* and *B* into a common environment, *i*. As is well known, somatic cell strains transferred to tissue culture will undergo considerable changes, including the resorption of much overt specialized equipment, yet at the same time show the following signs of the preservation of inherent differences. (1) They retain certain gross morphological and physiological distinctions, such as differences of size, of nutrient requirements, of growth rates, of viability, &c. (2) Returned to conditions appropriate for the restoration of specialized equipment, each strain will develop products characteristic of its own original type or a related type. (3) When being transplanted, after prolonged stay *in vitro*, into a foreign environment in a host organism, they will behave essentially according to the middle rather than the left diagram. (4) While in the *in vitro* environment, each strain may proliferate and give rise to countless descendant cells, all of which will bear the marks of the parent strain, hence likewise behave according to points (1), (2) and (3); that is to say, while *B* in small *i* will behave differently from *B* in *b*, and *A* in *i* differently from *A* in *a*, the differentials between *B* and *A* are preserved and passed on to their cellular progeny throughout the subsequent processes of growth and cell division without depletion or attenuation.

Consequently the apparent simplification, or as it is commonly called 'dedifferentiation', of cells in tissue culture signifies merely a loss of external criteria such as referred to in section II. 2, without loss of type-specificity. It does not imply reversion to a common type. The wider range of responses of cells kept in different media only proves the plurivalency of cells even in advanced stages of differentiation, rather than a recovery of omnipotency. To determine the breadth of this range, which varies from type to type and from stage to stage, is a purely empirical task. The crucial fact to remember is that this range undergoes progressive narrowing within each sector of the protoplasmic time-continuum. In conclusion, the true, that is inherent, properties which connote cytodifferentiation reside within the cell boundaries and are of such nature that they can be reproduced true to type in unlimited amounts during continued proliferation. No consideration of differentiation that confines itself to the cell individual can thus be complete. A complete account must include the prior history during which equipotential cells have acquired their properties of myoblasts, chondroblasts, nephroblasts, &c., respectively, as well as the subsequent history during which these various cell types can continue to reproduce each its own kind differentially even in an indifferent common medium.

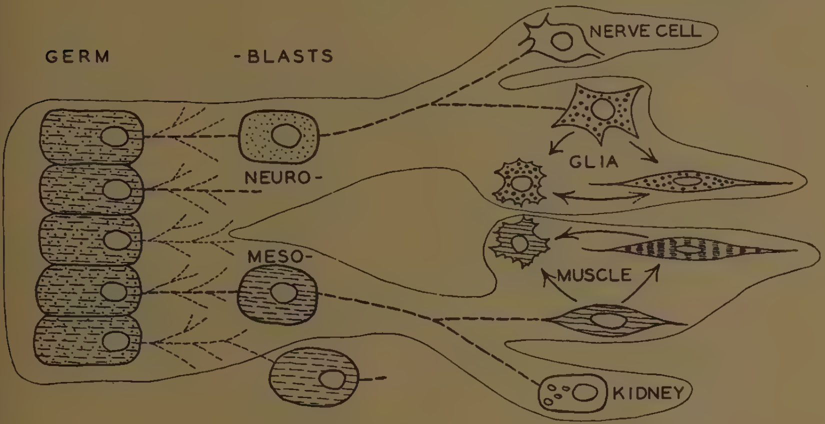


FIG. 2

These facts are summarized in the two diagrams, Figs. 2 and 3. In Fig. 2 the segregation of omnipotent cells of the early germ by a series of events into a neuroblastic and a mesoblastic strain is depicted, with the former branching into nerve cells and glia cells, the latter into muscle cells and kidney cells, among other specializations. This diagram takes into account that, even in advanced stages, cells can still assume a variety of expressions; for instance, both muscle and glia cells can appear as either spindle cells or macrophages (see arrows), which are functional conversions, called 'modulations', and indicate latitude of expression within a given type rather than instability of the type as such.

In Fig. 3 one particular cell line is singled out to show the transformations its protoplasm continues to undergo even after type specificity has been established. Stage *a* could represent a medullary plate cell or an epidermal cell maturing (with concomitant growth and division) into stage *b*, then passing on into stage *c*, at which there appears for the first time a separation into reproductive and non-reproductive groups. The reproductive ones may remain segregated in special 'germinal' layers or cords, such as the neural epithelium lining the brain ventricles or the Malpighian layer of the skin. Knowing that the former can no longer give rise to anything but neural cells and the latter to nothing but epidermal derivatives, it is hardly proper to call them 'undifferentiated' as is done in common usage. While the upper cell in stage *c* keeps turning out more cells of the particular type, the lower one has become a terminal cell individual. As such it produces additional specialized equipment of the sort usually used for its identification.

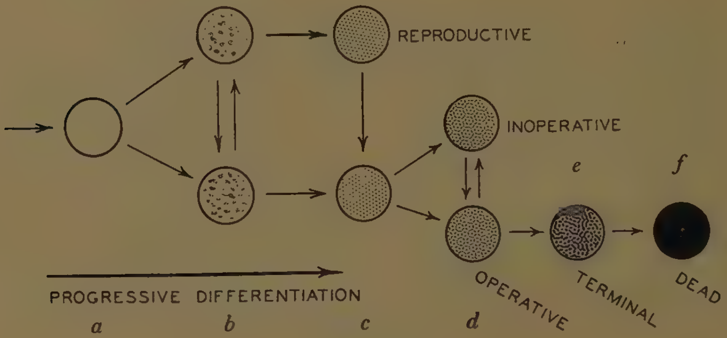


FIG. 3

It has frequently been asserted that there is a certain general inverse or antagonistic relationship between proliferation and terminal specialization. This rule, however, has by no means universal validity and would be weakened even more if instead of cell division the process of protoplasmic reproduction were used as criterion of growth. Neurons, for instance, keep growing throughout life yet hardly ever divide, and from what is known about cellular hypertrophy, one can make a similar case for other cell types. Perhaps the general absence of mitotic activity in terminal stages of differentiation is due to the diversion of materials or energy resources that would be needed for the mitotic apparatus into the building of specialized equipment.

Even the non-reproductive cells of stage *c* are not necessarily single-tracked. Depending on what type they belong to, they can go on to a variety of functional states. Most common among these are the alternatives of inoperative and operative phases which might be reiterative, either cyclically or aperiodically, or represent a singular event. Familiar examples are the active and inactive states of glands, the castrate or hormonally stimulated states of secondary sex characters, the fixed or mobilized forms of reticulo-endothelial cells, and the like. An operative cell which has become single-tracked may then assume a terminal expression (state *e*), as in the case of neuron or sensory cell, often eventuating in death, as for instance in keratinized cells of the horny layers, red blood cells, secretory cells or holocrine glands, &c. (stage *f*).

The course of events here schematized could be compared to the conveyor belt of an assembly line in an industrial plant in which a raw product is gradually transformed into the finished product. The analogy is correct in that, at every step of the process, additional factors must enter to permit it to proceed to the next step towards completion, and also in that the process may be arrested at any one stage. Thus many cells never reach the maximum possible terminal expression. Contrary to a machine assembly, however, the progress of cellular differentiation is marked less by the stepwise addition of new components than by the reorganization and selective rearrangement of existing ones within the system.

IV. WHICH PARTS OF THE CELL DIFFERENTIATE?

Having thus circumscribed the criteria and the nature of differentiation, let us now turn to the question of what parts of the cell take part in it. We may immediately exclude from our consideration elementary molecular constituents that travel freely between the cell and its environment, such as water, electrolytes, and most small organic molecules, and restrict our question to those organic systems which occur solely within the cell space. For simplicity we may lump them into five categories: (1) the genome; (2) the non-genic parts of the nucleus, or the nucleome; (3) the continuous cytoplasm; (4) the cytoplasmic inclusions, among which we may distinguish two kinds, (4*a*) those in common to a great variety of cell types, e.g. mitochondria, microsomes, the Golgi system; and (4*b*) specialized products peculiar of a given cell type, e.g. myofibrils, neurofibrils, secretion granules, pigment, &c.

The last class, (4*b*), obviously differs widely between different cell types. From what we have said before, particularly in section II. 3 and 4, this presupposes the existence of differential production machineries in the respective cytoplasms. Consequently, at least part of system (3) must be assumed to be subject to differentiation. As for the cell organs of class (4*a*), the answer is uncertain. Except for trivial differences of size, configuration, and density, they are usually considered as equivalent in all cells. It is quite possible, however, that beyond their universal functional similarities (e.g. concentration of respiratory enzymes on mitochondria, lipid character of the Golgi system, ribonucleic acid accumulation in microsomes), they show finer biochemical distinctions corresponding to their respective cell types. The extragenic nucleome (2) must be considered as differentiated, not only from general cytological appearances, but also because of the reported origin of certain specific cell products (e.g. secretions) within the nucleus. The genome (1), on the other hand, is generally assumed to retain its identity in all the various somatic cell types, at least as far as the quality of its composition is concerned. The evidence rests essentially on genetic data. Occasional attempts to connect differentiation with quantitative changes in the genome, such as polyploidy, can be discounted in view of (*a*) the normal cytological and histological differentiation of animals with haploid as well as polyploid (from triploid to octoploid) chromosome sets; (*b*) the regular occurrence in some tissues of cells with multiple chromosome sets (e.g. mammalian liver, insect scales) affecting solely cell size, but not basic cell character; and (*c*) a simple consideration of the very large number of qualitatively different cell types in the higher animals.

Even after narrowing the issue of differentiation to this point, it is still vague and intractable because we have recognized no further inner distinctions within the various subsystems (2) to (4) to which we concede differentiation, treating them as if they were homogenous substances of identical composition and properties throughout. In order to go beyond verbal generalities and to confront the living system with more realistic and analytical questions, we must try to form a more concrete idea of just what the microcosm of a cell really looks like, consists of, and how and by what forces it undergoes its progressive transformations. This calls for replacing the common notion that protoplasm is a 'substance' by a more realistic representation which takes into account its character as a 'system' composed of populations of molecular species of various properties and groupings, interacting with one another within the ordered facilities, as well as limitations, of the space they occupy. With this in mind, the molecular model of differentiation, presented in the following section, was constructed.

V. CYTODIFFERENTIATION IN MOLECULAR TERMS

Any attempt to formulate a molecular concept of cytodifferentiation is bound to remain, for the time being, highly speculative. However, the free flight of fancy can be considerably restrained by paying rigorous attention to certain principles of cell behaviour in differentiation that have been derived from countless observations and experiments. In an earlier publication I have labelled three of these basic principles as 'discreteness', 'exclusivity', and 'genetic limitation'. Discreteness of cytodifferentiation means that cell types fall into rather sharply delimited classes without intergradations. There are, for instance, no transitions between muscle cells producing actin and myosin, thyroid cells producing thyroxin, Langerhans cells producing insulin, and nerve-fibres producing myelin. This argument is not weakened by the fact that many cell types may contain or produce common components (for instance, collagen or melanin), much as all living cells must be able to reproduce common equipment for their basic physiological functions such as respiratory enzymes. But with regard to other parts of their endowments, different cell types differ radically. Therefore, the principle of discreteness and the absence of a continuous spectrum of transitions point clearly to the fact that differentiation between strains is based on qualitatively different chains of chemical reactions. The second principle, exclusivity, expresses the fact that a cell cannot follow more than one of the several discrete courses originally open to it, at a time. Once it has become definitely engaged in one course, alternative courses are automatically suppressed. Evidently the cell in differentiation behaves as a unit. This principle resembles the principle of complete dominance established in genetics. The third principle, genetic limitation, expresses the empirical fact that the various ontogenetic courses open to a given cell are strictly circumscribed by the genetic endowment of the species to which it belongs. Combined with the principle of discreteness, this means that the finite, although very large, repertory of reaction types of the various descendants of a zygote is strictly limited from the start by the chemical equipment of the genome.

Heeding these clues and from a general critical interpretation of ontogeny, we arrive at the following concept of differentiation. The genome of the zygote endows all descendant cells with a finite repertory of modes of reaction. What is commonly called 'differentiation potency' may be interpreted as a finite assortment of chemical entities. These entities, of course, must not be viewed as direct precursors of any final characters, but as a reactive system, the constant interaction of which with systems of the extragenic space will only gradually yield the later specific characteristics of the various cell strains. If we envisage the response repertory of a cell as a system of alternative chains of reactions permitted by the original genic endowment, then differentiation involves the selective triggering off of certain of these chains to the exclusion of others. Divergent differentiation between two cell types thus is due not to differences of native composition but to the activation of different parts of the common equipment.

The first divergent activations in a germ arise presumably from preformed regional differences in the chemical composition and configuration of the surface layer of the egg; for blastomeres enclosing one particular sector of this surface mosaic will confront their genome with a different reactive background than will those that have received another sector. The ensuing reactions, further diversified by interactions among neighbouring parts, lead to the next steps of activation from the still multivalent, if already somewhat restricted, reaction repertory, and so on down into the late stages pictured in Fig. 3, in a continuous sequence of interactions. Since the extragenic space, i.e. the genic environment, is thus undergoing progressive transformation, it is evident that every new reaction must be viewed in terms of the cellular system in its actual condition at that particular stage, moulded by the whole antecedent history of transformations and modifications, rather than solely in terms of the unaltered genes at the core. Incidentally, keeping this in mind ought to stop the confusing practice of labelling all intrinsic properties of a cell at an advanced stage as 'genetic', but those brought out by still later interactions with neighbouring cells or diffusible agents as 'environmentally' or 'hormonally' induced, forgetting that no cell develops independently, but that all of them have gone through a long chain of similar 'environmental' interactions with neighbouring cells and the products of distant ones.

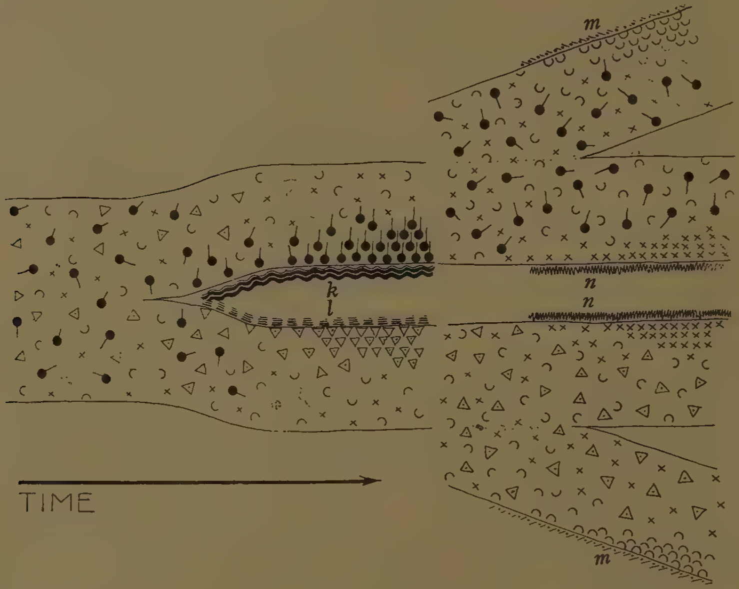


FIG. 4

This view of differentiation as a chain of chemical reactions is, of course, not new, but then it is not sufficiently tangible and specific either. It offers no model for the process of selective activation; no explanation of how, despite the great diversity of possible reactions, their systematic order in time and space, underlying functional organization, can be maintained; in general, it is too non-committal to guide deeper experimental penetration. In an effort to fill it with more specific meaning, I shall sketch in the following a concept the gist of which is incorporated in the diagram, Fig. 4.

The left end of the figure represents a sample of protoplasm (similar models apply to all subsystems listed above in IV. 2–4). We ignore chemical constituents of ubiquitous occurrence and concentrate on those molecular species characteristic of the particular cell. Though their building-blocks are of general occurrence, the peculiar pattern of their assembly into larger systems is unique and apparently reproducible only in the presence and with the aid of pre-existing similar patterns. In order not to encumber our model with unverified assumptions, we shall make no attempt to identify these molecular species chemically as to whether they are proteins, nucleic acids, lipids, polysaccharides, or higher-order combinations of such. Their number may be very large but we shall symbolize them only by four different representatives, depicted as crosses, triangles, crescents, and pins. If the chosen sample were part of the egg cytoplasm, these would be part of the primordial molecular endowment. If the sample represents a cell in a more advanced stage, this would be a derivative population, modified by the past phases of ontogeny.

It is characteristic of living systems that a state of random dispersion like that in the extreme left of the diagram would be unstable and gradually give way to orderly segregations effected and maintained presumably with the aid of metabolic energy and other factors to be mentioned presently. As I have outlined in earlier publications, the mixed molecular populations will sort themselves out according to the specific physical and chemical conditions that prevail in the different regions of the cell space; moreover, as a result of their different localizations, the segregated populations will reciprocally contribute to the establishment of similar ordering conditions for subsequent processes. The resulting organized behaviour of mixed molecular populations in the living cells, their ‘molecular ecology’, contrasts sharply with the behaviour of the same populations in the random dispersed state existing in homogenates. Only those chemical compounds in a cell are of relevance that are given opportunity either to interact or to alter the conditions for the interaction of others. Now it is generally recognized that the more complex a biochemical system is, the more subtle and specific are the prerequisites for its operation and maintenance. That is to say, in order for any one of the symbolic compounds of our model to have enough stability to be demonstrable, the conditions for its existence in that locality must be uniquely favourable. Such conditions may pertain to its synthesis or local accumulation or simply protection against breakdown and dissipation. They will include physical factors as well as chemical requirements such as the proper concentrations of constituent compounds, accessory factors, energy-yielding reactions, &c. Non-random distribution of key compounds thus signals the existence in different parts of the cell space of different sets of conditions favouring different types of reactions. Consequently, just as we have come to recognize in the visible range that morphological differentiation is merely an index of antecedent differentiating processes, so on the molecular scale we may now consider the demonstrable localization of given molecular species as merely an index of underlying physico-chemical conditions favouring either the reproduction or the accumulation of that particular species in that particular sample of protoplasmic space.

Among the conditions favouring selective molecular grouping, interfaces deserve special attention for the following reasons. Adsorption to interfaces can stabilize a molecular array against disruption by thermal agitation or liquid convection. Anisodiametric molecules at the same time will be adsorbed in a definite spatial orientation and may thus be ordered and aligned with the result that (*a*) if they are enzymes, their activity will be increased because of their closer packing and the common orientation of their active groups; and (*b*) if they are structural units, their orderly assembly into larger structures will be facilitated. Factors conferring this organizing faculty upon interfaces are not only differences of electric potential and the purely physical conditions favouring the formation of monomolecular films, but in a subtler sense, the chemical bonding between sterically matching or otherwise corresponding chemical compounds to either side of the interface. Compounds may thus be trapped in the interface by their affinity to sterically interlocking compounds already there. Although models of this type of interaction are currently popular as explanations of enzyme-substrate relations and antibody-antigen binding, and although their extension to phenomena of gene reproduction, protoplasmic replication, and surface interactions among cells seems highly suggestive, our model is independent of any such special interpretation. All it assumes is that a condition *k*, indicated in the upper branch of the diagram by a wavy line, representing a certain physical and chemical constellation along that interface, favours the selective accumulation from the interior of the pin-shaped compounds, whereas the condition *l* prevailing in another interface, symbolized by the broken line in the lower branch, promotes the adsorption and concentration of the triangular species.

Thus the same protoplasm, faced with two different conditions, will acquire surfaces composed of radically different compounds, hence qualitatively different. Let us call such populations which have assumed singular controlling positions 'master populations'. To explain subsequent development they must satisfy two demands—first, they must have a governing influence on the further reaction pattern of the cell, and second, they must stimulate the reproduction of their own kind. Qualitative effects on metabolism by border populations are indicated by the fact that (*a*) they can control selectively the substance traffic between the system and its medium in the way of a 'living membrane'; (*b*) if endowed with enzymatic activity, potentiated by their ordered state, they will catalyse characteristic chains of reactions; and (*c*) as structural elements, they constitute foundations for the anchoring and stacking up of other selected compounds (see later). Moreover, if these master compounds in the surface, either directly or through some of their derivative effects, were to monopolize certain metabolic resources to the exclusion of potentially competing compounds, which do not assume equally favourable positions, the latter would gradually be starved out and disappear irretrievably. This is indicated in the model by the dotting and later omission of triangles in the upper branch, and pins in the lower branch, of the diagram.

According to this model, the earliest steps towards differentiation involve primarily changes in the *disposition* of existing compounds, some of them being shifted to, and concentrated in, preferred interfacial positions. Only in further consequence, and with the passing of time, will their controlling functions in these preferred positions entail changes in the substantial *composition* of the systems to which they belong. This distinction between disposition and composition is fundamental, as the former is reversible, whereas the latter is not. So long as their contents in molecular species have not changed, two systems, even though they may have displayed different segments of their molecular populations in master positions, hence manifested different morphological and physiological aspects, can still be returned to a common equivalent state, if the respective key species can be dislodged from their controlling positions. Such reshuffling of the molecular population may come about as the result of solvatization, protoplasmic streaming, or other unstabilization of protoplasm; for instance, following either transfer of a cell into a foreign medium or changes in the inner milieu, as in inflammation or other pathological states.

On the other hand, once a given selected master species of molecules has occupied controlling positions long enough to have caused the competitive depletion of certain other species, then obviously a return to a common condition is no longer possible even though the key compounds may still be displaced from their controlling positions and others be induced to occupy their places. This condition is exemplified by the right-hand part of the diagram. In this the two molecular populations of the upper and lower branches of the left half of the diagram are assumed to have been first thoroughly stirred up by some factor mobilizing the cellular content and then confronted with new conditions, one symbolized as *m* and the other as *n*. Surface condition *m* traps the crescent molecules while the cross molecules aggregate in the *n* surface. Since both of the original cell types still possess both crosses and crescents, their reactions to conditions *m* and *n* are similar, as will be recognized by comparing the two inner branches and the two outer branches in the right half of the figure with each other. It should also be noted, however, that these resemblances do not connote identity and that the erstwhile differences in composition between the strains derived from the upper and the lower branches of our original protoplasmic strain have persisted. The rearrangements indicated in the right half of the diagram are the molecular version of the cellular phenomena previously described as modulations. They represent adaptations of cells to different conditions without change of fundamental equipment, but also without implying that the cells of different strains, which have undergone similar or parallel adaptations in response to identical media, have thereby become constitutionally alike.

According to our model, all irreversible differentiations can be said to arise by way of an initial reversible modulation. The other principles of cellular differentiation listed before can likewise be readily translated into terms of this model. The principle of discreteness is the result of the presence in the molecular repertory of the particular protoplasmic strain of a limited number of discrete key compounds that can assume controlling master positions. The principle of genetic limitation reflects the fact that the number and character of these key species is determined by the genic endowment of the zygote. The principle of exclusivity expresses the complete dominance in the determination of consecutive cell transformations by the molecular master species selected for surface occupancy over other molecular species not so favoured, hence excluded from exercising a determinative role.

Ordering processes of this type, spreading from an interface into the interior, are instruments of progressive organization. They are agents of selective activation and specific communication by which a given surface state can gradually evoke a conforming response from the enclosed parts. It should be borne in mind that in this progressive interaction all other interfaces can act as sites of selective screening and conversion according to their own molecular occupancy, so that one cannot take it for granted that any complex chemical entity can pass through a series of such boundaries (nuclear surface, surface of chromosomes, genes, cytoplasmic particles, &c.) without major modifications. In reverse, the actions thus activated within each enclosed system can alter conditions in the whole hierarchy of systems in ascending order. Accordingly the process we have modelled here crudely for a single protoplasmic fragment must be envisaged as repeating itself in manifold variations as each system interacts with its adjacent space. In this light, one could ask whether what we normally call 'activation' of certain components of the genome in cellular differentiation does not likewise consist of the selective segregation into controlling or active master positions of the appropriate fraction of the molecular repertory of the genes.

In conclusion, this model epitomizes the broadest statement that can be made about the biochemistry of the living cell in its organized state, as contrasted with its homogenized or disorganized condition, namely, that what determines the activities of the system is not the totality of chemical compounds it contains but the specifically selected assortment of compounds that have an opportunity to interact or otherwise operate, this being only a relatively small fraction of the total. Thus knowledge of the content of a protoplasmic system is of interest only in that it limits the possibilities of what can happen. However, in order to know just what will happen in a given case requires knowledge of just what part of the content will be placed in the appropriate conditions where it can operate. This is merely another and more explicit description of the property we usually refer to as 'organization'. To take it into account, biochemistry will have to develop a special field of 'topochemistry'.

We have assumed in this model that, in order for a protoplasmic strain to undergo divergent differentiation, the two branch lines have to be exposed to specificially different external conditions k and l. Similar branching points will arise later leading to further subspecialization. Some such dichotomies may be merely in the nature of modulations, as in the divergent expressions in media m and n. In modulation, the particular type of organization will last only as long as the respective conditioning environments are actually present, whereas in true differentiation a permanent residue of the response to a particular environment has become fixed in the cell so that it can continue itself even in the absence of the organizing environment. The descendants of a modulating cell may, of course, carry permanent and irreversible characters reflecting the particular state of the mother cell during which they were procreated.

Clearly the tacit assumption underlying this model has been that the original protoplasmic system, unless subjected to either condition *k* or *l*, would have remained stationary and unchanged. However, the validity of this assumption is open to question. It may be doubted whether any living system, even when left entirely to its own devices in a stable environment, could remain unaltered over prolonged periods of time. Slow progressive changes are known to occur in unfertilized eggs as well as in 'ageing' protozoan and metazoan cells. Moreover, any sequence of protoplasmic transformations that greatly outlasts the duration of the condition that has set it off will give the appearance of autonomous intrinsic change.

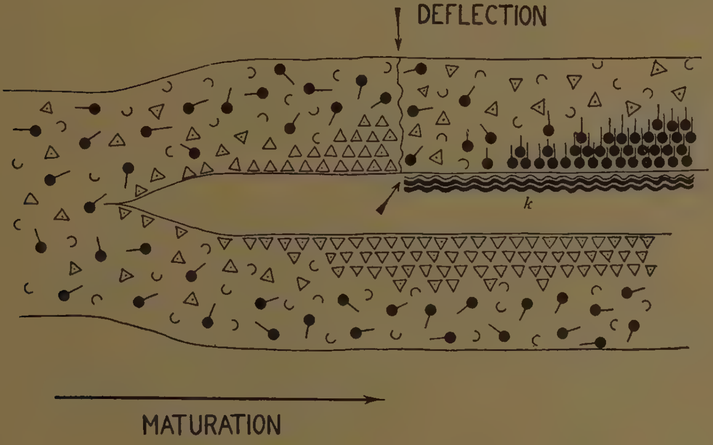


FIG. 5

This being the case, our model may have to be amended in the sense of Fig. 5. The lower branch shows what would happen to the protoplastic system if subject to no additional differentiating influences. It can be seen that a progressive segregation of molecular species occurs, but in this case 'autonomously', that is, by virtue of a course of events initiated much earlier in the cell's history. This course, however, can now be deflected into a different direction by the appearance of a single differentiating condition *k*, provided this new condition (*a*) remobilizes the molecular populations, (*b*) dislodges the master species of triangles, and (*c*) replaces it by a master population of pins with greater affinity (meaning perhaps better steric conformance) to the inducing category *k*.

According to this model, divergent differentiation within a given cell strain would require exposure not to two different sets of conditions but to only one, while the other would simply continue an intrinsic pattern of 'maturation'. Let us quote some examples. Divergent differentiation of secondary sex characters has often been described as the switching of a neutral cell form into either the male or the female direction by corresponding sex-differentiating factors, including hormones. This implies double-switch action. On the other hand, it has also become clear that in many forms of animals the differentiation of one type of sex character is actually identical with the assumed neutral condition, with the opposite sex development being actively enforced by appropriate hormonal deflexion from the original course. This then is single-switch action. Similarly, when tapetum of the developed urodele eye proves capable of reproducing retinal cells of the optic layer, but not vice versa, one could interpret this to mean that tapetal differentiation represents the autonomous course from which the cells would have to be positively diverted in order to produce retinal derivatives. There are innumerable examples in experimental embryology and pathology amenable to similar interpretations. The two variants of our model, corresponding to Figs. 4 and 5, are summarized in Fig. 6*a* and *b*, mainly to show that in practice the decision between them may often be difficult to make, particularly if the branching points, that is, the events causing divergencies, follow each other in quick succession. At any rate, these are empirical questions, and the models are merely intended to help phrasing them in a realistic light.

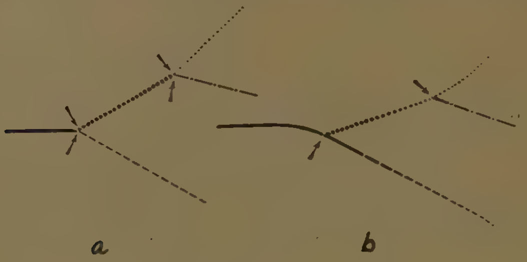


FIG. 6

VI. HOW IS THE DIFFERENTIATED STATE PERPETUATED AND TRANSMITTED DURING GROWTH?

Our models depict the progressive transformations of a given molecular population in time, but they do not take into account the continued increase of this population, which we call growth. Since the symbolic molecules used in our models are by definition peculiar to the particular type of protoplasm, hence are found only inside the cell, the mechanism of their reproduction must be looked for entirely within the cellular system. Despite the splendid upsurge of work on protein synthesis and particularly on the role of nucleic acid systems in cell growth, we still lack the major keys to the understanding of just what goes on in protoplasmic reproduction. It may be well, therefore, to outline certain basic considerations which any future theory of somatic growth must take into account.

1. Specialized cell protoplasm of a given cell strain can continue to propagate its own kind, as set forth in section III.

2. There is no evidence that this reproduction of type-specific protoplasm can be referred to the existence of corresponding type-specific differentiations among the genomes of the various somatic cell strains (see above, section IV).

3. To reconcile these two points, one might assume that during differentiation the capacity for self-reproduction or self-replication has been conferred upon some of the molecular key species that distinguish different cell strains according to our models.

4. The autonomy, that is independence from the genome, of such hypothetical self-reproducing cytoplasmic units is contradicted by experimental results in protozoans and yeasts. As for somatic cells, actual observations on suitable objects have made even the very concept of self-reproducibility highly questionable. Our own demonstration of the fact that neurons are in perpetual growth and that this growth proceeds solely from the nuclear territory of the cell space, supported by the recent cytochemical work on the substantial role of the nucleus in cytoplasmic synthesis, indicates strongly that the actual production sites in the process of growth are located in the nuclear territory. Therefore it is not unlikely that the so-called self-reproducing cytoplasmic particles derive the substance for their 'growth' essentially preformed from the nuclear (or more restrictedly, genic) space, hence represent stations for the deposition and possible type-specific conversion, rather than for the synthesis, of the basic protoplasmic compounds. Their growth thus would be by accretion.

5. Considering the fact that the extragenic nuclear space ostensibly undergoes differentiation (see section IV), it would be equally plausible, of course, to assume that the conversion of primordial genic products into type-specific variants occurs already within the nucleus itself.

An effort to bring these various considerations to a common denominator leads to the following integrated concept. The various specialized high-molecular key-compounds in the cytoplasm would not really possess the faculty of catalysing the synthesis of more of their own kind from elementary constituents, as implied in the concept of 'self-reproduction', but would only have the role of models in the reshaping or converting of more complex primordial compounds. furnished from the genic space, into conforming patterns. For this reason these model compounds may be given the purely descriptive name of 'templates'. By imposing their pattern upon other compounds, they would perpetuate their kind without being themselves involved in the process of multiplication. They therefore have the faculty of 'self-perpetuation', not self-multiplication. Evidently, if the type-specific master compounds which we have assumed to characterize differentiated strains act in this template fashion, the continued reproduction of the particular type of protoplasm would be ensured. Myoplasm would engender more myoplasm, nephroplasm more nephroplasm, neuroplasm more neuroplasm, and so forth, despite the identity of the genome.

In passing, it may be pointed out that this concept, suitably expanded, also furnishes the clue for the harmonious growth relations between different parts of the same organism and for their automatic regulation upon disturbance. This growth control, explained more fully in previous publications, operates on the basis of the following premises: (*a*) Some of the master compounds selectively sorted out in different strains act as models for their own multiplication and thus for the perpetuation of the strain. (*b*) Production of primordial genic (species-specific) compounds in each growing cell is superabundant. (*c*) The rate of their conversion into type-specific protoplasm is proportional to the number of extragenic master compounds free to act as templates. (*d*) Complementary compounds combining with templates render the latter ineffective, hence inactivate or veritably sterilize their template function. (*e*) Compounds of such complementary combining power are being thrown off as by-products of the type-specific remodelling process. In a very crude picture we might visualize them as the chips coming off as a primordial compound is whittled down to the shape of the template model in the replication process. Again with a purely descriptive term, we might call these small units of a configuration complementary to the templates 'antitemplates'. (*f*) Because of their small size, the antitemplates can diffuse from the cell and pass freely between the cell and its exterior, whereas the templates, because of larger size or conjugation, remain confined to the inside. (*g*) By virtue of the diffusion gradient between their intracellular production sites and the large extracellular liquid space in blood, lymph, and tissue juices, the antitemplates will leave the cell at a given rate. (*h*) As their concentration in the outside medium increases and the gradient flattens, the rate of diffusion from the intracellular to the extracellular space will decrease, until finally equilibrium is reached. Correspondingly, as their cellulifugal diffusion falls off, their relative intracellular concentration increases. (*i*) Since the proportion of free templates to inactivated templates in the intracellular space will decrease as the concentration of antitemplates inactivating them increases, the reduction in the rate of outward diffusion of antitemplates will automatically produce a reduction in the number of free template molecules, hence, according to (*c*), an automatic retardation in the reproduction of the type-specific protoplasm which we measure as growth. Whether this, in turn, rebounds on the rate of primordial genic synthesis or merely switches the utilization of the primordial products from the reproduction of basic protoplasm to the elaboration of specialized cell products, thus accounting for the observed interference of cell proliferation with specialized cell function, is problematical. Further complications are introduced into this scheme by the consideration that in the intracellular space both templates and antitemplates will be metabolized, whereas in the extracellular space the antitemplates will be katabolized without resynthesis, which presumably would cause a steady cellulifugal drift.

It can be readily seen that this system permits all protoplasm of a given kind, however widely dispersed throughout the body, to retain intercommunication and regulate its total growth. Let us consider, for instance, that part of a given type of growing protoplasm is artificially removed, thus reducing the production of templates and antitemplates of that particular type by a given amount. To the residual part of the body the loss of templates will not be perceptible since, as strictly intracellular entities, they had not been in circulation. The only information of the changed situation will come from the sudden drop of the corresponding antitemplate species in the extracellular pool. As a result of this drop of extracellular concentration, the rate of their diffusion from all residual cells of the same kind will increase, thus leaving uncovered intracellular templates for renewed growth catalysis. This will register as an automatic spurt of growth in all tissues having the same characteristic as the removed one. We have experimental evidence to show that this is at least a major part of the mechanism of so-called compensatory hypertrophy and compensatory hyperplasia.

A second way by which to reduce the effective concentration of a given antitemplate population in the extracellular pool, hence to cause an automatic growth response in the homologous cell types, is to release into the extracellular space free templates from their intracellular confinement. As these combine with their specific antitemplates in the pool, the intra- to extracellular concentration gradient of antitemplates will steepen and their growth-inhibiting effect will be correspondingly diminished. Thus, injury to a tissue, by bringing cell content into circulation, will have the same effect on homologous tissues as has partial removal. On the supposition that the 'growth-promoting' effect of embryonic extract in tissue culture is due precisely to this mechanism, we have obtained experimental support of highly suggestive, if not yet fully conclusive, nature. Even stronger support has come from experiments carried out in the embryo itself in which organ growth can be influenced in the expected direction by spilling cell content into the vascular or extra-embryonic spaces.

Whether the templates and antitemplates are to be conceived in terms of steric fitting, like antibodies and antigens, is wholly conjectural and by no means crucial for the scheme as here presented. The envisaged mechanism views the organism as a vast system of chemically intercommunicating differentiated protoplasts. Communication by special hormones appears as merely a more highly adapted and specialized version of this more general principle, the difference being that hormones are specialized cell products turned out in terminal cell phases (section II. 1 and 2), whereas we are here concerned with the underlying cell substance itself (section II. 3 and 4).

VII. THE PRODUCTION OF STRUCTURAL ORDER

Differentiation in molecular terms implies unscrambling and selective localization of molecular populations, setting the stage for consecutive reaction chains. The result is over-all structural order. We are satisfied that thermodynamically the production and maintenance of a non-random condition requires the constant input of energy but the actual mechanisms by which chemical processes are translated into orderly physical structure are for the most part still obscure. They are ostensibly of many diverse kinds, each to be subject to separate analysis in its own right. A few common examples are presented in the following.

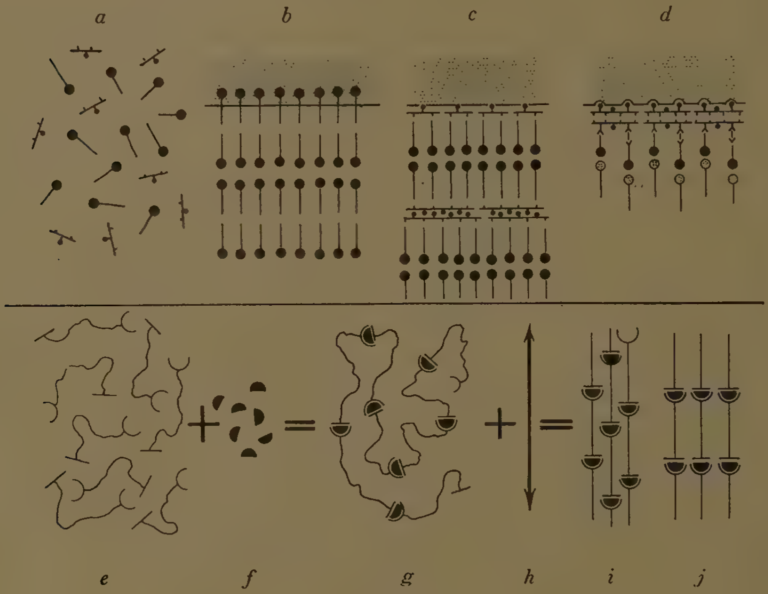


Fig. 7

One is based on the ordering effect of interfaces. It starts with the formation of a monomolecular film of oriented molecules, yielding a first-degree planar order, which then constitutes the floor plan, as it were, for a higher degree of order attained by the orderly stacking up of additional layers in the third dimension. The simplest cases are schematized in the upper part of Fig. 7. Let us assume a population of two molecular species in random dispersion (Fig. 7*a*), the pin-shaped kind with a lyophilic group at one end and a hydrophilic group at the other, and a water-soluble kind, e.g. a certain protein. If only the former were present at an oil-water interface, oriented molecular fixation of the kind shown in Fig. 7*b* would take place. In a more general sense, we may replace the oil-water system by any diphasic system with regard to which different end groups of the same molecule would show differential selective affinities. In the presence of the second species, the mixed population could sort itself out according to the diagram, Fig. 7*c*. Current concepts of the cell membrane and of the myelin sheath of nerve-fibres support this model. By introducing additional molecular species we can construct more complex systems such as in Fig. 7*d*, in which the third dimension no longer shows repetitive structure as in *b* and *c*, but displays qualitative variety. It is important to keep in mind that the adsorbed surface population maintains its stable arrangement in the face of convections and thermal agitation of an otherwise liquid system. Through the stacking on of additional layers the stable organized crust gains in width and may become microscopically distinct as gelated exoplasm. If this is true of the cell surface, then similar processes must also be conceded to the interfaces along genes, chromosomes, nucleolar and nuclear surfaces, and particulates in general.

In its application to the primordial differentiation of the egg, this scheme implies that if the egg surface contains a mosaic of molecular species of different properties segregated in different sectors, this topographical pattern would retain its stable localization despite the movements of the egg content during cleavage or the experimental reshuffling by centrifugation or stirring. It therefore provides firm bearings for the subsequent changes in individual blastomeres as outlined above in section V.

Differences are thus initiated in different parts of the original protoplasmic mass which may not become effective or manifest until at a much later stage. Yet as soon as differences appear, they present an emergent condition of further differentiation for the various interacting cell strains, for the condition of any one cell is at the same time an environmental factor for its adjacent cells. In consulting our model, Fig. 4, it is clear that if conditions *k* and *l*, after having produced differential effects in the respective cells, subside and the two cells come in apposition, each constitutes a new outside condition for the other. Depending on the state of consolidation or responsiveness reached, either cell may now react to the other by a further step of transformation. The interaction is mutual, yet whether or not a response will materialize is a matter not only of the presence or absence of adequately responsive units but also of the degree of mobility and displaceability.

One readily recognizes in this a general model of processes of induction by cellular contact. As I have indicated on earlier occasions, the neuralization of ectoderm by subjacent cell layers, or layers of organic molecules deposited on its surface, might be a case in point, although the evidence that this type of induction is of transmissive rather than transportative nature is by no means conclusive. On the other hand, in the case of lens induction by contact with the retinal layer, signs of cellular orientation signalling molecular regrouping of the requisite kind have been observed.

A second example of typical unrandomization of protoplasmic components is found in the formation of linear complexes with definite orientation in space, best illustrated by the fibrous proteins but presumably applying to all kinds of elongate and anisodiametric molecules. A simple sequence of steps is represented in the lower half of Fig. 7. In *e* we see constituent molecules with specifically configurated end groups; the addition of molecules of fitting properties (*f*) will link the original elements into chains (*g*). Polymerization and coacervation *in vitro*, blood clotting, protoplasmic coagulation, &c., furnish examples for such linear compounding. The resulting chains (*g*) are linear but not straight. Save for true crystallization and the formation of tactoids, such filaments require extraneous vectors in order to be straightened out. Such vectors (*h*) may consist of physical tensions, convection currents, and perhaps strong electrostatic fields. The resulting arrangement (*i*) leads to the building up of straight fibrils, fibrillar bundles, and fibres by progressive condensation. A still higher degree of order is produced in those cases in which, in addition to the lengthwise alignment, the constituent elements fall in lateral register (*j*), producing cross-banding, as in chromosomes, collagen fibrils, myofibrils, &c.

These examples may suffice to prove the diversity as well as the intimacy of interrelations between physical and chemical factors underlying the establishment of spatial order which we call structure. They are important not only because of their conspicuous contributions to the microscopic features of terminally differentiated cells, but because of their less overt ordering function at all stages of the differentiation process.

VIII. THRESHOLDS AND STATISTICAL ORDER

Problems of differentiation are commonly dealt with in terms that tacitly imply identity among the cells within a given cell population. In reality, no two cells are ever exactly alike, and random variations during ontogeny are apt to magnify rather than reduce initial inequalities. This being the case, a given cell population, subject to an inductive or otherwise differentiating influence, will give a uniform response in all its elements only if that influence exceeds a certain critical magnitude; corresponding to what in neurophysiology is known as a supramaximal stimulus, or in nutrition as superabundant food-supply. If, however, the influence is of lower magnitude, intensity, or duration, only a given fraction of the elements of the population will respond, namely, those of sufficiently low thresholds to be affected by the given dose of action. This is explained by the diagram, Fig. 8. In the left portion the frequency distribution of the elements of a cell population according to thresholds of responsiveness to a given agent is plotted, assuming that they vary at random (normal distribution curve). The abscissae represent stimuli of increasing dosage while the ordinates (upper base line) represent percentages of cells beginning to show effective response at the particular dosage level. The sigmoid curve (lower base line) represents the integral of the distribution curve, hence its ordinates give the total number of elements activated at any given dosage level. As the middle portion of the integral curve is nearly a straight line, the number of activated elements increases within the median range in almost linear proportion to the stimulus. Some proportionality between stimulus and response is thus to be expected on purely statistical grounds.

(In terms of the model, Fig. 5, we might view the threshold condition as the one in which the deflecting factor *k* can attract a sufficient number of the pin-shaped molecules into master positions to give them the edge over the competing triangular type.)

Let us assume now that the dosage of a differentiating influence is of the magnitude indicated by the heavy black line. This corresponds to an ordinate value of 25 per cent. That is to say, the probability is that the cell population will contain, on an average, 25 per cent. cells sufficiently sensitive to respond to this given stimulus condition. Evidently this probability will be the same for any size or density of the population. Sample results are depicted in the middle panels of Fig. 8 for three cell populations differing in densities in ratios of 1 : 2 : 4. Although the number of constituent elements varies, the ratio between responding units (black) and refractory units (white) remains the same. In other words, the relative proportions of the two segments of the population are determined although it is impossible to predict for any given unit whether it will respond or not. Statistical regularities of this kind are doubtlessly involved where the stationary composition of a cell population is to be insured throughout growth, multiplication, and repair.

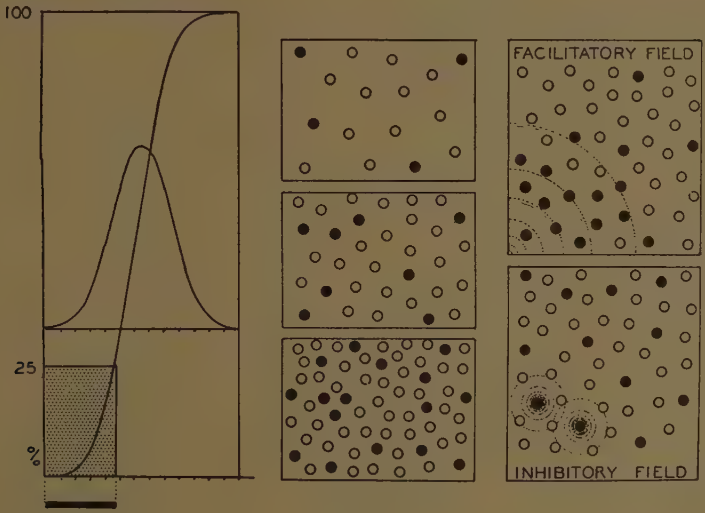


FIG. 8

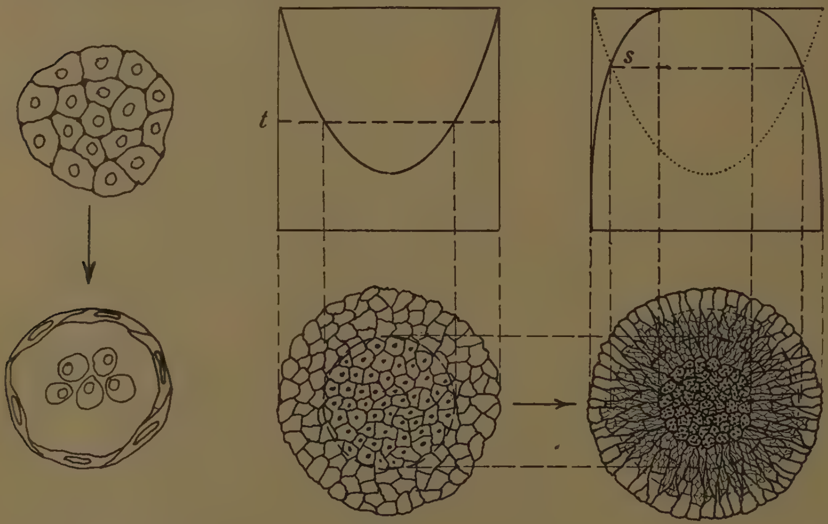
Similar numerical constancy, however, can also be obtained by an altogether different procedure, namely, by precise cell lineage with differentiating divisions.

Such precision regulation has been suggested, for instance, for the constant ratio between scale-forming cells and ordinary epithelial cover cells in butterfly wings.

Statistical dichotomy of differentiation is further modified in those instances in which elements which have responded to a given stimulus thereby become the seat of secondary actions which either facilitate or inhibit similar reactions by their neighbours. These effects are illustrated in the right-hand part of Fig. 8. The top panel illustrates the case of a responding element spreading an action which lowers the threshold of other elements. As a result, the responding cells will appear grouped in clusters. Conversely, as is indicated in the right bottom panel, a spreading influence which raises thresholds will inhibit a similar cell response within a given radius, entailing thus a higher degree of regularity of distribution.

IX. FIELD EFFECTS

These considerations lead over immediately to a section of great morphogenetic significance, namely, 'field' responses, in which the fate of a given cell is determined by the position of the element within the group. Geometric position evidently signifies tangible physico-chemical constellations at that particular site.



(*a*) (*b*) (*c*)

Fig. 9

Examples of this type of interdependence are too common to require listing in this place. As one of the simplest cases, let us take the fate of an embryonic blood island in the chick embryo as indicated in Fig. 9*a*. A group of equipotential cells (top) undergo divergent differentiation (bottom), the outer ones forming a vascular endothelium, and the inner ones free blood cells. The terms 'outer' or 'inner' refer not to properties intrinsic to the component cells but to differentials conferred upon them by virtue of their position within the group. If the original cluster (top) were to be rearranged or bi-sected, or if individual cells simply traded places, each cell would then behave according to its actual, rather than its former, position.

Systems of this kind show the simplest form of field behaviour. Their mode of operation can be conceived of as follows (Fig. 9*b*, *c*). Let us assume a simple equipotential cell cluster like the one in Fig. 9*a* (top), bordering on either cells of some other type or on some other medium. As a result of interaction along the free surface, a certain course of reactions will be activated in the cells that share in the surface. Let us assume that this entails the release from those cells of substances or activities that spread inward with a gradient from their source. In addition, we might assume that the whole cell mass is engaged in activities producing metabolites which can diffuse into the outer medium, hence will tend to be most concentrated in the centre. Additional factors of this kind would be crust-core differentials in accessibility to essential food constituents, oxygen supply, and the like. The resultant gradient of these composite conditions is indicated in the top part of Fig. 9*b*. Let us now assume that the cells of the group can react to a particular constellation or combination of such factors by a differentiating step according to our model, Fig. 5, and that their thresholds for this reaction lie at the level of the broken line *t*. Then in the central area, within which the mentioned group factors exceed in intensity the requisite threshold minimum, all cells will be switched into the altered course. Whereas in the preceding section we have considered differentials arising within a homogeneous stimulus field by virtue of random variation among cells, the present case demonstrates the response to a graded field of partly self-engendered stimuli. In the transition zone near the threshold level, the prospective response of a given individual cell will again be unpredictable, but the steeper the gradient, the sharper will be the line of demarcation.

A new chain of responses now having been activated in the central cell group (indicated by dots, Fig. 9*b*, bottom), let us assume that this activity entails two further events, namely, first, a contraction of the central group (from the broken to the solid contour) and second, the release by them of new reaction products into the vicinity. As a result of the former, the outer cells attached to the shrinking core will be subject to centripetal tension, hence become radially elongated, as shown in Fig. 9*c* (bottom), which in turn may facilitate the outward diffusion or transmission of the newly produced agent. Its emergent diffusion field is represented by the solid curve in Fig. 9*c* (top). Let us assume further that the earlier substance gradient has persisted (dotted line) and interacts with the new centrifugal factor so as to form an impervious precipitate which will stop further diffusion, and that this happens in the area where the concentrations of the two agents are about equal, as indicated by the intersection of the two curves at the level *s* (broken line). As a result, a new zone intermediate between the original core and the new barrier will arise with new influences, activating in the constituent cells the next step of their differentiation repertory. In this manner, complexity within the originally equipotential group increases progressively according to a definite orderly pattern of organization in which 'position effects' substitute for the various extraneous media *k*, *l*, &c., of our models.

It is easy to imagine that by the linking of such spherical fields with linear fields and the further introduction of asymmetries, highly complex products can be obtained. It is also to be noted that by combining such a gradient concept of activating factors with the above molecular concept of discrete differential cell response, the conversion of merely quantitative differentials into qualitatively diverse responses finds a ready formal explanation. On the other hand, too many abstractions of this kind have been accepted in the past as 'explanations' prior to and without tangible verifications. They are presented here, therefore, primarily as guides to further analysis and as a hypothetical framework within which proper experiments can be designed. This cautioning note is not to support the scientifically untenable contention, heard on occasions, that a formal operational analysis in dynamic symbols is valueless unless accompanied by a precise identification of the substantial nature of the agents involved. This would be like saying that perfectly valid laws of optics cannot have been developed, as they have, in ignorance of the electromagnetic nature of light. Considering current trends, it would indeed seem more appropriate to caution against the illusion that the mere identification of physico-chemical systems can have much explanatory value unless their formal order of operation in the living system has likewise been revealed. It is of greater pragmatic value to set forth at least formal models of phenomena known to occur in living systems than to ignore or even deny the occurrence of those phenomena just because our present incomplete, oversimplified, and elementary schemes cannot account for them. Field phenomena in supercellular systems are a firm reality to all those observers and analysts of living phenomena who have not deliberately confined themselves to the investigation of elementary and fragmentary processes in which field properties can be legitimately ignored. At the same time, little has been done to instil more concrete content into the various field concepts. The remarks in this last section merely constitute one small and crude effort towards concreteness.

X. CONCLUSIONS

As indicated in the beginning, the study of differentiation stands to make much faster progress if instead of looking for sweeping generalizations or insisting on generalities, which by the very nature of the differentiation process can have little practical meaning, we first disassemble the complex process into its constituent components, many in numbers and of diverse kinds, and then accord each a separate and systematic study in its own right; and if, furthermore, in doing this, we visualize the real objects as concretely as our knowledge and reasoning will permit us, instead of operating solely with formal and symbolic notions as heretofore. The models introduced here for the purpose of concretization are undoubtedly far too simple and incomplete. Yet they may have the merit of pinpointing the targets at which we shoot our questions and direct our experiments on 'differentiation'.

The reader will recognize that many of the current discussions on virus reproduction, plasmagenes, adaptive enzymes, and somatic 'inheritance' in their relations to the differentiation process can be well accommodated in the terms of the concepts here advanced. However, in view of the narrative character of this article, no literature has been quoted. The main earlier publications of the author referred to in the text as precursors of the present discourse are as follows:

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Regulation and Differentiation within Field-districts in Imaginal Discs of *Drosophila*

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GENITAL imaginal discs of third instar larvae of *Drosophila* have been cut into medial, paramedial, or transverse parts of different sizes. The fragments were then implanted into the body cavity of a host larva. This medium provides the implants with ample nutritional material and hormones. The fragments thus differentiate their imaginal structures simultaneously with the metamorphosing host. A detailed analysis of the respective differentiations of the various parts has led to the following results and revealed the following properties of the different blastemata which are embodied in a genital disc (Hadorn *et al.*, 1946, 1949, 1950).

1. The building materials of the different parts (elemental organs) of the genital apparatus can be localized within the larval disc and a schematic map can be designed which indicates the positions of the presumptive areas (districts) which give rise either to sperm pumps, claspers, anal plates, spermathecae, or vaginal plates.

2. The parts of the imaginal discs develop only their district-specific elements. Overlapping regulations, leading to a differentiation characteristic of a neighbouring district, have not so far been observed. The more or less homogeneous appearing cell mass of a larval disc represents therefore by no means a harmonious equipotential system, but must be considered as a mosaic of districts, each of which contains definite and circumscribed developmental potencies.

3. Each district, however, has the properties of an embryonic field (cf. Weiss, 1939) with full capacities of regulation for the developmental pattern of its district-specific elemental organ. Thus parts of these field-districts are able to restore the normal organization of an intact field and to differentiate the field-specific structure of normal shape and normal size. After being cut into three parts, an imaginal disc is, for instance, capable of differentiating three pairs of anal plates instead of only the one pair which the same blastemic material would have formed if incorporated into an intact disc.

4. The regulation in parts of field-districts, at least in *Drosophila melano-*

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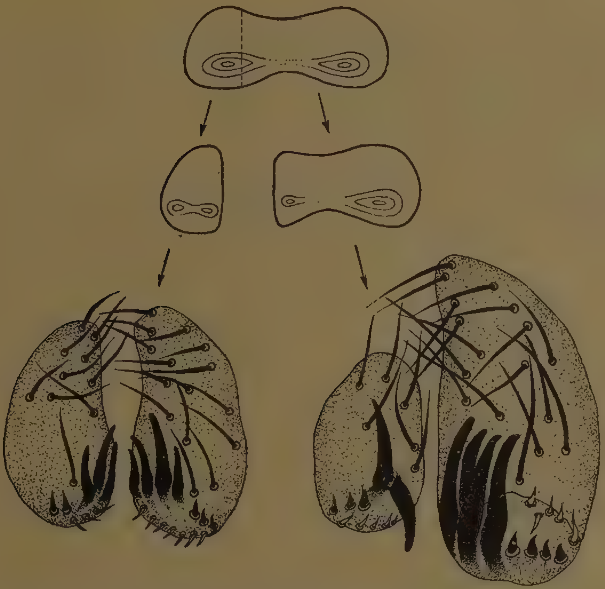
gaster, is always accompanied by an extra increase in the number of cells. Hence the mitotic activity must continue until the normal cell number, characteristic of a whole field-district, is reached. By counting the number of bristles in anal plates or in claspers, it was shown that, regardless of whether the material of a genital disc forms two or six plates, we find for each group the same mean value of bristle numbers per plate and only plates of normal size. Similarly, each of the two halves of a sperm-pump field forms a sperm pump which is as big and as complete in its complicated bilateral morphology as the single sperm pump of a normal genital apparatus. Thus the processes involved in the regulative activities in parts of fields appear as 'all-or-none effects'.

5. Recently we found (unpublished work carried out in collaboration with Dr. P. S. Chen) that the decision as to whether regulation in a transplanted field-part does or does not take place depends to a large extent on the time interval which elapses before the host larva enters metamorphosis. Sagittal halves of female discs, all of the same developmental stage, were implanted into hosts of different ages. In one series, where the hosts began to metamorphose 2–6 hours after the implantation, only 45 per cent. of the sagittal halves formed two spermathecae, while the others did not regulate. According to the prospective value of half a field they differentiated only one spermatheca. In another series the younger hosts entered metamorphosis 18–24 hours after the implantation. Here, 70 per cent. of the halves showed full regulation by forming two spermathecae of normal size.

In cases where an 'all-or-none effect' occurs, the regulative process depends apparently on a sufficient enlargement of the field-district by mitosis. In younger hosts there is more time and material at the disposal of the implants for the extra cell divisions, whereas in older hosts the disc cells fall too soon under the influence of the metamorphosing hormones which force the implant into immediate differentiation.

6. A type of regulative behaviour different from that characteristic of the fields for anal plates, sperm pumps, or spermathecae in *Drosophila melanogaster* was found by Hadorn & Chen (unpublished) in the field-districts for anal plates in males of *Drosophila montium*. In this species the anal plates differentiate a very conspicuous pattern consisting of four different bristle types: 4–5 giant thorns, 3–5 thorns of medium size, 6–9 small thorns arranged in a row at the ventral margin of the plate, 17–22 slender hair-like bristles which occupy the middle and the dorsal surface of the plate. The accompanying figure shows the characteristic result of an experiment where a genital disc has been cut paramedially into a large ($\frac{3}{4}$) and a small ($\frac{1}{4}$) part. Without cutting the disc would have formed only two plates. In our experiment each of the parts has differentiated a pair of them. But the plates are of distinctly different sizes. How are we to interpret this result? Based on the experiences gained on *Drosophila melanogaster* (Hadorn, Bertani, & Gallera, 1949) we assume that the presumptive material for anal plates is organized as a bicentric field-district. After being cut

into two, a new bicentric organization becomes established in each one of the parts. But contrary to the similar experiments in *Drosophila melanogaster*, there is either no or insufficient enlargement of the reorganizing field fragments. Nevertheless a regulation does take place. Otherwise we would expect to find differentiation of some incidental fragmentary sectors of anal plates. But actually whole plates are formed with all four bristle types, arranged according to the normal pattern. Thereby the number of bristles in the different types becomes reduced in a proportional manner. It seems that here an 'image of the pattern' can be reproduced in distinctly different sizes. Cellular differentiations then must be regarded as secondary to the 'moulding matrix' of the field structure.



Differentiation of male anal plates in parts of a genital imaginal disc of *Drosophila montium*. The position of the bicentric field-district for anal plates and its reorganization after cutting is indicated above. Each separated part differentiated a pair of anal plates. It can be concluded that the large plate to the right, which is the only one of normal size, was built by the cell material of the intact right field centre. The three other plates originated from the blastema of the divided left field centre. All of them are harmoniously reduced in size and in number of bristles. Thereby the complete pattern is maintained.

7. Treating the imaginal discs of *Drosophila melanogaster* in vitro with colchicine or with NaCl solutions of different concentrations induced characteristic alterations of field-specific differentiations. Colchicine, for instance, acts in such

a way that instead of two spermathecae up to ten are formed by one treated field. It might be that this poison acts as a 'chemical knife' which disorganizes the original single field so that a series of new field centres arises. Each of them would then behave like a part isolated in an experiment in which the field has been fragmented by real cutting.

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Über Zelldifferenzierung im Integument der Insekten und ihre Bedingungen

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DIE besonderen Beiträge, die das Integument der Insekten zu den Fragen der Zelldifferenzierung bietet, beruhen wesentlich auf der *Vielzahl verschiedenartiger Differenzierungen*, die bei dieser hochentwickelten und dabei doch kleinwüchsigen Tiergruppe auf einzelne große Spezialzellen verteilt oft auf engem Raum *nebeneinander* vorkommen, oder auch, in der Metamorphose durch Häutungen die sonst meist verborgene Fähigkeit der Zelle zu wiederholten Differenzierungsleistungen demonstrierend, *nacheinander* von denselben Zellen hervorgebracht werden. Ein weiterer charakteristischer Zug in der Entwicklung des Insektenintegumentes ist ihre Abhängigkeit von einer Serie *hormonaler Anstöße*, in deren Gefolge nach jeder Häutung zunächst eine Mitosenperiode, danach die Herstellung einer neuen Kutikula stattfindet und schließlich die Häutungsdrüsen in Tätigkeit treten, die beim Vollzug der nächsten Häutung mitwirken. Im Lauf der Entwicklung verändert sich nun die Zusammensetzung des jeweils wirksamen Hormonsystems, und damit, allein in Abhängigkeit von ihm, auch die Differenzierungsleistung des Integuments. So bringt das *Grundepithel* eines holometabolen Insekts unabhängig von seinem Alter und seiner Vorgeschichte je nach dem hormonalen Milieu, dem es ausgesetzt wird, entweder eine larvale oder eine pupale oder schließlich eine imaginale Kutikula hervor (Piepho, 1950, 1951). Im normalen Entwicklungsgang erfährt es also eine zweimalige *Umdifferenzierung*. Daneben können im Epithel regionale Unterschiede in der Höhe der Schwelle gegenüber bestimmten hormonalen Faktoren bestehen und ein *Muster* aus verschiedenen nebeneinander auftretenden Kutikularbildungen erzeugen (Piepho & Heims, 1952). Besonders empfindliche Anzeiger sowohl für die hormonalen wie auch für andere die Zelldifferenzierung bestimmende Einflüsse finden sich aber unter den für die Insekten charakteristischen, in das Grundepithel eingefügten *Kleinorganen*, die als Borsten, Schuppen, Drüsen oder Sinnesorgane aller Art jeweils aus wenigen, zum Teil einzeln unterschiedlich differenzierten und zu einer bestimmten Größe heranwachsenden Zellen zusammengesetzt sind (Henke, 1947, 1951).

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In der besonderen Entwicklungsweise dieser Organe tauchen nun noch andere die Zelldifferenzierung berührende Fragen auf, denn sie entwickeln sich aus jeweils einer einzelnen *Stammzelle*, die ähnlich einer Eizelle des sogenannten determinativen Entwicklungstypus in einer streng festgelegten Folge *differentieller Zellteilungen* schrittweise in die für unterschiedliche Differenzierung und Teilfunktion vorgesehenen *Spezialzellen* des Organs zerlegt wird (Abb. 1 *a–c*). Kommen einfache Vermehrungsteilungen einzelner Zellen hinzu, so ist ihre Anzahl gering und gleichfalls gesetzmäßig fixiert (Abb. 1 *d*, *e*). Wenn ein solches

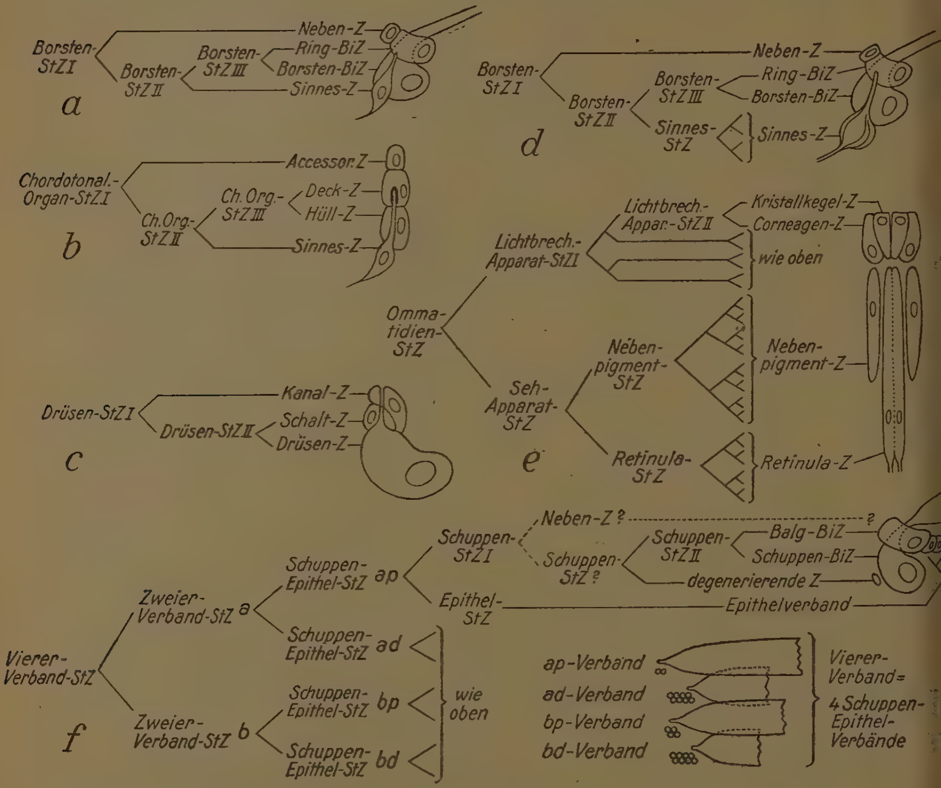

ABB. 1. Zellteilungsfolgen in der Entwicklung von Kleinorganen im Integument der Insekten. *a*, Sinnesborste mit einer Sinneszelle (Henke & Rönsch, 1951). *b*, Chordotonalorgan (E. Jägers, noch unveröffentlicht). *c*, Versonsche Drüse (Henke, 1951; R. Stabenau, noch unveröffentlicht). *d*, Sinnesborste mit 4 Sinneszellen (Henke & Rönsch, 1951). *e*, Ommatidium (Bernard, 1932). *f*. Schuppen-Epithel-Verbände der Schmetterlinge jeweils in Vierzahl aus einer Viererverband-Stammzelle hervorgehend (Henke & Pohley, 1952). Unten rechts Beispiel eines Viererverbandes mit verschiedenen der möglichen Kombinationen von Schuppengröße und Epithelzellenanzahl. — *Bi Z*, Bildungszelle; *St Z*, Stammzelle; *Z*, Zelle.

Zellsystem nicht erst am Ende der Entwicklung sondern schon in der Embryonalzeit oder auf einem späteren Durchgangsstadium fertiggestellt ist, so können die von seinen chitinbildenden Gliedern hervorgebrachten kutikularen Bestandteile bei den folgenden Häutungen mit der Kutikula des Grundepithels abgeworfen und ähnlich wie sie von denselben Zellen jeweils erneut hergestellt werden, sei es in dem gleichen Zustand, oder, weil die Bildungszellen noch weiter wachsen, in vergrößerter Form, schließlich auch, wie etwa bestimmte Borsten bei den Entwicklungsstadien der Wachsmotte *Galleria mellonella*, in einer mehr oder weniger stark abgeänderten Gestalt (Kruminš, 1952). Wie das Grundepithel können also auch die Bildungszellen, offenbar unter hormonalem Einfluß, eine *Umdifferenzierung* erfahren, und zwar geschieht es nun hier mit Sicherheit *ohne Zwischenschaltung von Zellteilungen*. Auch ein Vorgang, der wie die Schuppenbildung der Schmetterlinge normalerweise nur einmal beim Abschluß der Entwicklung stattfindet, kann im Experiment wiederholt werden, und mit großer Wahrscheinlichkeit vollbringen auch hier einzelne Bildungszellen eine zweimalige Differenzierungsleistung (Piepho & Meyer, 1951).

In der Entwicklung der *Spezialzellen in Sinnesborsten- und Schuppenapparaten* sind in neuerer Zeit durch die von verschiedenen Untersuchern mit unterschiedlichen Methoden zutage geförderten Ergebnisse eine Reihe von Entwicklungsmechanismen neu oder besser als bisher erkennbar geworden, die teils im *Vollzug der Zelldifferenzierung*, teils in ihrer *Vorbereitung* und *Lenkung* wirksam sind.

Das erste mikroskopisch sichtbare Anzeichen einer bevorstehenden Borsten- oder Schuppenbildung ist *ein mit Endomitosen verknüpftes gesteigertes Größenwachstum der Bildungszellen* oder auch schon einer ihrer Vorfahrenzellen (Henke & Mertz, 1941; Henke & Pohley, 1952). *Gegenüber den Faktoren, welche die spezifische Zelldifferenzierung bestimmen, ist dieses Größenwachstum ein relativ unabhängiger Prozeß.* So können bei Schmetterlingen im Gefolge eines bei der Raupe eingeleiteten Regenerationsvorganges Epithelzellen, Schuppen- und Balgbildungszellen gleichermaßen vergrößert sein, ohne daß sich ihr jeweils besonderer Differenzierungscharakter verändert (Kühn, 1949; Kühn & Piepho, 1940). Bei der Wasserwanze *Corixa punctata* vergrößern sich die Borstenbildungszellen und ihre Kerne anhaltend während der ganzen Larvenzeit, und entsprechend werden auch nach jeder Häutung größere Borsten als nach der vorhergehenden gebildet. Eine Endomitose tritt aber in den einzelnen Bildungszellen nicht in jedem Häutungsintervall ein, sondern immer nur dann, wenn das Größenwachstum vorher eine bestimmte, für jede Kernstufe charakteristische Schwelle überschritten hat (Ch. Lipp, im Druck). Ist das bei einer Bildungszelle nicht der Fall, so machen ihre Chromosomen eine *Pseudoendomitose* durch, indem sie, ohne sich zu teilen, die in den Funktionszyklus der Zellen eingepaßten Bewegungen und Formänderungen der in einer Endomitose begriffenen Chromosomen anderer Bildungszellen mitmachen (Ch. Lipp, im Druck). Zwar hat es den Anschein, daß schon zu Ende der Embryonalentwicklung die Anzahl der einer Zelle bevorstehenden Endomitosen sich in der Dicke der Chromosomen abzeichnet, doch werden *die einzelnen Endomitoseschritte jedenfalls durch das ihnen vorausgehende Größenwachstum ausgelöst. Andererseits bestimmen nun die Endomitosen das Ausmaß des Wachstums*, denn wenn eine Endomitose eintritt, so wird der Größenzuwachs des Kerns gegenüber den ohne Endomitose vollzogenen Schritten jeweils bis zu einer bestimmten Grenze gesteigert. Diese *Grenze* ist ebenso wie die in der Auslösung einer Endomitose erkennbare *Schwelle* für jede Polyploidiestufe charakteristisch. Beide zusammen sind ein Ausdruck für *bevorzugte Gleichgewichtslagen zwischen Kerngröße und Chromosomenanzahl*, wie sie aus der verbreiteten, bisher aber wohl noch nicht in derselben Weise wie hier analysierten Erscheinung des *rhythmischen Kernwachstums* bekannt sind. Bei der Borstenbildung der Dipteren werden statt der endomitotisch entstandenen Reihen von Polyploidiestufen der Bildungszellkerne *Riesenchromosomen* von unterschiedlicher Größe hergestellt (Schwenk, 1947), die sich auf verschiedene Anzahlen von *Kryptoendomitosen* (Bauer, 1938) zurückführen lassen. Der *Größenzuwachs des Kernvolums* beim Übergang von einer Kernstufe zur nächsthöheren kann bei der Mehlmotte *Ephestia Kühniella* bis zu einer Verdoppelung gehen (Henke & Mertz, 1941). Bei *Corixa*, deren Chromosomen viel lockerer im Kernraum verteilt liegen als die der Schmetterlinge, ist er mit einem Vergrößerungsfaktor von im Mittel 1·76 wesentlich geringer. Im Vergleich hierzu ist der *Zuwachs des Zellvolums*, wenn man es nach der Größe der von der Bildungszelle als lang auswachsender Fortsatz hergestellten Schuppe oder Borste bestimmt, unverhältnismäßig stark, und so ist auch die mit den Polyploidiestufen der Bildungszellkerne verknüpfte Größenvariabilität etwa bei den Schuppen der Schmetterlinge viel ausgeprägter diskontinuierlich als bei den Kernen selbst, so daß man die Kernstufen aus den bevorzugten Größentypen der Schuppen leichter als aus den Kerngrößen bestimmen kann. Bei den Borstenbildungszellen von *Corixa*, deren annähernd kegelförmige Produkte eine einigermaßen zuverlässige Volumbestimmung erlauben, ist der *Vergrößerungsfaktor des Zellvolums gleich der 3. Potenz des für das Kernvolum gültigen Wertes.*

Will man hiernach die Borsten- oder Schuppenbildung eher als Sekretions- wie als Wachstumsleistung der Bildungszellen ansehen, so handelt es sich bei der *Differenzierung der Borsten und Schuppen* jedenfalls um die Herstellung eines mehr oder weniger kompliziert geformten Sekretes, das auch mit der Chitinisierung, Pigmentierung und Erhärtung noch erhebliche chemische Veränderungen erfährt. Lees und Picken haben die Entwicklung normaler und mutanter *Borsten* von *Drosophila* unter diesem Gesichtspunkt studiert und die normale Gestalt und Beschaffenheit der Borste in allen wesentlichen Zügen aus den *Eigenschaften des Bildungsmaterials*, speziell der Orientierung der Molekülketten des Chitins, sowie aus seiner in einer Reihe von Schritten jeweils von bestimmten *Genen* kontrollierten Herstellung interpretieren können (Lees & Picken, 1945). Ähnlich wie die mannigfachen von Mutationen bewirkten Abänderungen können auch die jeweils in bestimmten Entwicklungsstadien durch extreme Temperaturreize ausgelösten *Modifikationen* verschiedene Phasen im Ablauf der Borstendifferenzierung kenntlich machen (Henke, v. Finck & Ma, 1941; Ma, 1943). Da hier sicherer als bei den Mutationen der Zeitpunkt anzugeben ist, in dem eine bestimmte abnorme Entwicklung einsetzt, wäre es im Hinblick auf die Frage der Wirkungszeiten verschiedener Gene nützlich, die auf den beiden verschiedenen Wegen hervorgerufenen Reihen von Entwicklungsstörungen genauer zu vergleichen. Bei den *Schuppen* der Schmetterlinge ist der Aufbau der Gesamtform allein aus der sekretorischen Tätigkeit der Bildungszelle und den molekularen Eigenschaften des Chitins wohl noch nicht ohne weiteres zu verstehen. Die Schuppe erhält ihre charakteristische Form schon vor Abschluß des Wachstums und der Chitinisierung (Stoßberg, 1938). Dabei konnten durch Modifikationsexperimente auch hier verschiedene schrittweise nacheinander die endgültige Formbildung bestimmende Prozesse erfaßt werden (Köhler & Feldotto, 1937; Kühn, 1948), von denen nun einer, wie Kühn gezeigt hat, den Unterschied zwischen dem Stiel und dem freien Ende lediglich als *polare Organisation* festlegt. Die Ausmodellierung des freien Endes mit seinen charakteristischen Zacken findet erst später statt. Schließlich, wenn die Formbildung abgeschlossen ist, wird die Schuppe noch annähernd maßstabsgetreu wesentlich vergrößert.

Die Borsten und Schuppen entstehen jeweils in engem Kontakt mit einer zweiten Bildungszelle, die ihre Basis kragenartig umfaßt und eine sie beweglich festhaltende Chitinstruktur herstellt, den Borstenring oder Schuppenbalg (Abb. 1 a, d, e). So erhebt sich die Frage nach den *Entwicklungsbeziehungen zwischen den Bildungszellen der Borste oder Schuppe und ihrer Haltevorrichtung*. Die bisher vorliegenden Beobachtungen lassen einerseits eine gewisse *Autonomie*, andererseits aber auch bestimmte *Abhängigkeiten* erkennen. So kann nach den Ergebnissen von Kühn (1948) ein im wesentlichen normal gestalteter Schuppenkörper ohne Kontakt mit einem Balg entstehen, doch ist dabei das normalerweise vom Balg umfaßte Stielende mehr oder weniger deformiert. Bei *Drosophila* können nach Lees & Waddington (1942) gewisse mutativ bedingte Störungen der Borstenform auf ein abnormes Lageverhältnis zwischen Borsten- und Ringbildungszelle zurückgeführt werden. Andererseits ist wieder, nach denselben Untersuchern, die Ringbildung als solche der Borste gegenüber autonom, denn sie kann, wenigstens wenn abnormerweise zwei oder mehrere Ringbildner vorhanden sind, ganz ohne Borstenbildungszelle vor sich gehn; es entsteht dann ein einheitlicher Ring. Solche zusammengesetzten Bildungen können nun auch eine Borste einschließen, und es wäre interessant, in derartigen Fällen die Maßverhältnisse genauer zu prüfen. Bei *Ephestia* und ähnlich auch bei der Trichoptere *Limnophilus flavicornis* können Schuppen oder Borsten verschiedener Größentypen jeweils mit Haltevorrichtungen gepaart sein, deren Bildungszellen verschiedene Kernstufen aufweisen (Henke & Mertz, 1941; G. Rönsch, noch unveröffentlicht), doch variieren bei statistischer Betrachtung die Kernstufen der beiden aus einer gemeinsamen Stammzelle hervorgehenden Bildungszellen gleichsinnig, und bei *Ephestia* besteht über diese summarische Abstimmung hinaus eine enge Korrelation der Balgweite zu dem Größentypus der Schuppe, die auf eine unmittelbare Abhängigkeit zwischen den beiden Bildungszellen schließen läßt (Henke, 1945).

Verfolgt man nun die Entwicklung der Borsten- und Schuppenapparate von dem Größenwachstum und der Differenzierung ihrer Bildungszellen rückschreitend weiter, so ist zunächst zu fragen, worauf der *differentielle Charakter der letzten Stammzellenteilung* beruht, warum also die beiden mit ihr auftretenden Zellen sich unterschiedlich, die eine zum Borsten- oder Schuppenbildner, die andere zur Bildungszelle einer Haltevorrichtung entwickeln, und wodurch der besondere Charakter der Entwicklung sowohl der einen wie der anderen Zelle bestimmt wird. Der Entwicklungsunterschied könnte schon innerhalb der Mutterzelle vorbereitet sein, so daß bereits die Teilung als solche inäqual ist und damit determinierend wirkt. In der Differenzierung pflanzlicher Zellen spielen, wie besonders Bünning (1952) gezeigt hat, solche inäqualen Zellteilungen eine bedeutende Rolle. Andererseits könnte die unterschiedliche Entwicklung aber auch erst den aus einer äqualen Teilung hervorgehenden Schwesterzellen aufgeprägt werden, etwa dadurch, daß sie infolge der Einstellung der Spindel unter verschiedene jeweils spezifisch determinierende Außeneinflüsse geraten. Die Untersuchungen von Lees & Waddington (1942) an Mutanten von *Drosophila* zeigen zunächst, daß *der differentielle Charakter der Teilung mehr oder weniger vollständig verloren gehen kann* und daß *die Entwicklung als Ring- oder Borstenbildungszelle nicht notwendig streng alternativ* ist, da statt der Borste auch Übergangsformen bis zu einer reinen Ringkomponente auftreten können, die sich dann mit dem Produkt der Schwesterzelle zu einer einheitlichen Ringfigur vereinigt. Ferner ist die abnorme Entwicklung mancher Mutanten deutlich mit einer abnormen Lage der Bildungszellen im Epithel verknüpft. So fanden sich bei Borstenausfall in der Tiefe des Epithels liegende und dabei ihrem Aussehen nach offenbar nicht entwicklungsfähige Zellen, und bei Formen, die Doppelringe ausbilden, zwei oberflächlich nebeneinander liegende Bildungszellen. Die Untersucher rechnen offenbar wesentlich mit einer *Bestimmung der Entwicklungsrichtung der Zellen durch ihre Lage im Epithel*, womit denn der leider bei den Mutanten nicht direkt untersuchten *Spindelstellung* eine entscheidende Bedeutung zukäme. In der normalen Entwicklung ist die Spindel bei Borsten und Schuppen allgemein mit großer Regelmäßigkeit schräg zur Oberfläche geneigt, und zwar so, daß die Bildungszelle der Borste oder Schuppe tiefer als die der Haltevorrichtung zu liegen kommt (Henke & Rönsch, 1951; Stoßberg, 1938). Andererseits sprechen mikroskopische Bilder dafür, daß tatsächlich bereits *die Teilung als solche auf Grund einer schon in der Stammzelle vorhandenen Differenzierung inäqual* ist. Eine damit bewirkte Determination der Tochterzellen könnte wohl eine Tendenz zum Aufsuchen bestimmter Lagen einschließen. Auch ein von der Ringbildungszelle ausgehender Einfluß von der

Art einer komplementären Induktion kann im Spiel sein. Es ist sehr wohl möglich, daß die Entwicklungsrichtung, die eine Bildungszelle einschlagen soll, mehrfach gesichert ist.

Von dem einen oder den beiden *vorhergehenden differentiellen Teilungsschritten, die an der I. Organstammzelle ansetzen,* ist wenig bekannt. Es wäre denkbar, daß es sich bei der für gewisse Mutanten von *Drosophila* charakteristischen *Vermehrung der Ring- und Borstenbildner* nicht oder nicht nur um überzählige Teilungen sondern um eine abnorme Entwicklung der in diesem Entwicklungsabschnitt normalerweise gebildeten Zellen handelt. Die Sinnes- oder *Sinnesstammzelle* wird wohl immer bei *senkrecht zur Oberfläche gestellter Spindel* nach innen zu abgegeben. Bei der Abgliederung ihres Korrelates in der Entwicklung der Schmetterlingsschuppen, der *degenerierenden Zelle,* zeigt das mikroskopische Bild eine deutlich *inäquale Teilung.*

Die Bestimmung einer Epithelzelle zur Organstammzelle kann in der embryonalen wie in der postembryonalen Entwicklung stattfinden. Über das Material, das zu der Frage nach dem Ursprung dieser Bestimmung für pflanzliche Objekte vorliegt, gibt die auf eigenen und fremden Untersuchungen fußende Diskussion von Bünning (1952) eine gute Übersicht. Bei den Insekten sind es häufig bestimmte *morphologisch ausgezeichnete Punkte,* an denen einzelne Epithelzellen des Integumentes den Charakter von Organstammzellen annehmen. Vielfach erscheinen die Stammzellen aber auch in bestimmten Feldern in einer mehr oder weniger regelmäßigen *rhythmischen Anordnung* (Henke, 1948*b*). Die Entstehung einer solchen Ordnung ist in vielen Fällen wohl am besten im Anschluß an eine von Wigglesworth (1940) entwickelte Vorstellung so zu verstehen, daß in einem bestimmten Entwicklungsstadium alle Zellen grundsätzlich gleichermaßen aber mit zufallsmäßig verteilten geringfügigen Geschwindigkeitsunterschieden die Tendenz zur Annahme des Charakters einer Stammzelle erwerben, und daß nun eine Konkurrenz um einen nur begrenzt verfügbaren Hilfsfaktor einsetzt, deren Selektionswirkung nur einer Auswahl von ungefähr gleichabständig verteilten Zellen die Weiterführung der angebahnten Entwicklung gestattet.

Für die bei der jungen Puppe zuerst sichtbar hervortretenden *Schuppenstammzellen der Schmetterlinge* gilt nun aber ein ganz anderes Prinzip (Henke, 1948*b*). Hier wird das *Epithel* normalerweise in der Anfangszeit des letzten Raupenstadiums *auf hormonalem Weg in die zur Schuppenbildung führende Entwicklungsrichtung eingelenkt,* unbeschadet der ungefähr um dieselbe Zeit auf gleiche Weise eintretenden Bestimmung zur Herstellung der Puppenkutikula. Im Transplantationsexperiment kann derselbe Entwicklungsgang auch schon in der Haut von Eiräupchen eingeleitet werden (Kühn & Piepho, 1940). Eine noch vor der Mitosenperiode des letzten Raupenstadiums auftretende erhöhte Strahlenempfindlichkeit des Epithels ist vielleicht der erste Ausdruck des neuen Zustandes (Henke & Pohley, 1952). Weniger wahrscheinlich ist wohl ein Zusammenhang mit der ebenfalls im Strahlenversuch nachweisbaren, gleich nach der letzten Larvenhäutung beginnenden Vervielfältigung der selbständig mutationsfähigen Einheiten in den Chromosomen, welche die erst wesentlich später einsetzenden Mitosen vorbereitet (H. J. Pohley, im Druck).

Im Lauf dieser letzten larvalen Zellteilungsperiode laufen nun an allen Zellen der genauer studierten Flügelanlagen zunächst etwa vier einfache Vermehrungsteilungen der Epithelzellen ab. Sie sind, wie das Verhalten einer bestimmten Mutante zeigt, für die normale Schuppenbildung wenigstens zum Teil entbehrlich (Querner, 1948). Dann folgen *drei differentielle Teilungen, deren letzte jede Zelle der Flügelanlage in eine Schuppenstammzelle und eine nur Epithelzellen liefernde Epithelstammzelle zerlegt* (Henke, 1946; Henke & Pohley, 1952; Abb. 1*f*). Der Zeitpunkt dieser Teilung konnte durch Strahlenversuche im Endabschnitt der Mitosenperiode festgelegt werden. Wird im Transplantationsexperiment der Entwicklungsabschnitt, in dem sie stattfindet, übersprungen, so wird die Schuppenbildung verhindert (Piepho, 1947). *Ohne sie kann eine Zelle offenbar nicht zur Schuppenstammzelle werden.* Sie ist *wahrscheinlich inäqual*, denn zwischen den beiden aus ihr hervorgehenden, qualitativ unterschiedlich sich entwickelnden Zellen besteht weiterhin eine eigentümliche Entwicklungsabhängigkeit, die am einfachsten mit der Annahme einer von Fall zu Fall wechselnden Aufteilung eines in allen Zellen der geteilten Generation, den Schuppenepithelstammzellen, in gleicher Menge oder Wirkungsstärke vorhandenen *Wachstumsfaktors* zu deuten ist. Die Epithelstammzelle macht nämlich umso weniger Vermehrungsteilungen durch, je stärker die später aus der Schuppenstammzelle hervorgehende Schuppe und ihr Balg heranwachsen, *und die Anzahl der von der Schuppenstammzelle durchlaufenen Endomitoseschritte bildet mit der Anzahl der Teilungsschritte der Epithelstammzelle eine konstante Summe.* Man kann von einem bei der Bildung der Schuppenstammzellen wirksamen *Kompensationsprinzip* sprechen, das die Schuppengröße, die Wachstumsordnung des Epithels und schließlich auch das Schuppenstellungsmuster, soweit es nicht durch möglicherweise mitwirkende Zellbewegungen bestimmt wird (Süffert, 1937), in Abhängigkeit voneinander hält.

Wie der Ausgleich zwischen den Bestimmungsfaktoren für Schuppenwachstum und Epithelzellenvermehrung bei der Teilung der Schuppenepithelstammzellen im Einzelfall eingestellt wird, hängt nun von den *beiden vorhergehenden Teilungen* ab (Abb. 1*f*). Die vier Schuppenepithelstammzellen des mit diesen Teilungen geschaffenen *Viererverbandes* können sich bei der folgenden, die Schuppenstammzellen liefernden Teilung mehr oder weniger *ungleich* verhalten — hierin prägt sich der in wechselndem Grad differentielle Charakter dieser Teilungen aus—und sie können außerdem *gleichsinnig* mehr oder weniger große Schuppen und entsprechend kleinere oder größere Epithelverbände liefern. Dabei herrschen bestimmte Gesetzmäßigkeiten, die am besten mit der Annahme eines nach einem einfachen Muster regional ungleichmäßig verteilten, vor Einsetzen der differentiellen Teilungen vielleicht im Zusammenhang mit der Bestimmung des Epithels zu imaginaler Entwicklung auftretenden Agens zu deuten sind (Henke, 1951; Henke & Pohley, 1952). Man muß dann annehmen, daß dieses vorläufig kurz als *Schuppenbildungsfaktor* bezeichnete Agens mit zunehmender Wirkungsstärke bei den beiden ersten differentiellen Teilungen in steigendem Maß ungleichmäßig verteilt wird und weiterhin bei der dritten die Schuppenstammzellen bei der Verteilung des konstanten Wachstumsfaktors in gleichfalls zunehmendem Grad begünstigt. Wenn die Zurückführung der in der Gestaltung des Schuppenkleides erkennbaren Gesetzmäßigkeiten auf die wechselnde Wirkungsstärke eines durch die Zellgenerationen hindurch weitergegebenen Schuppenbildungsfaktors das richtige trifft, so verlaufen auch die beiden ersten differentiellen Teilungen *inäqual.* Gewisse durch Strahlenwirkungen auslösbare Veränderungen liegen im Sinne einer örtlichen Erhöhung seiner Wirksamkeit (H. J. Pohley, noch unveröffentlicht).

Bei den *Trichopteren* hat sich in eingehenden Untersuchungen *keinerlei Hinweis auf eine den Verhältnissen bei den Schmetterlingen ähnliche Entstehungsweise der Borstenstammzellen* finden lassen (G. Rönsch, noch unveröffentlicht). Sicher ist schon die Entwicklung verschiedener zu einer einheitlichen Funktion nötiger Zellen aus einer gemeinsamen Organstammzelle ein abgeleiteter Zustand. Bei den Trichopteren ist es offenbar hierbei geblieben, während in der Entwicklung der *Schmetterlinge* zur Imago das *Prinzip der differentiellen Zellteilung* auf noch frühere Zustände ausgedehnt worden ist. Es hat hier *außer der Entfaltung auch die Herstellung der einzelligen Organanlagen* übernommen, und schließlich legt es, noch früher wirkend, *die räumliche Ordnung der Organe sowie gewisse quantitative Differenzierungsunterschiede* zwischen ihnen schon vor der Aussonderung ihrer Anlagen fest.

Tatsächlich geht die Bedeutung, welche dies in der Entwicklung des Schuppenkleides gewonnen hat, noch weiter als es hier deutlich gemacht werden konnte, denn die beiden ersten differentiellen Teilungen üben außer den geschilderten unmittelbaren Wirkungen auch noch einen Einfluß aus auf die *Fähigkeit der Bildungszellen, auf bestimmte in regionalen Gliederungen auftretende Faktoren anzusprechen*, wie sie sich etwa im Farbmuster zeigen (Henke, 1948*a*, 1948*b*), oder auch in zum Teil bedeutenden Strukturunterschieden wie dem zwischen Schuppe und Haar (H. J. Pohley, noch unveröffentlicht). Mit dieser *Verflechtung zwischen unabhängig voneinander durch ganz verschiedene Mechanismen erzeugten Differenzierungsordnungen* zeigt sich hier am Schuppenkleid der Schmetterlinge zuletzt noch ein Prinzip, das bei der Herstellung der unübersehbaren Mannigfaltigkeit von Differenzierungsunterschieden, wie sie die organische Entwicklung kennzeichnet, sicher ganz allgemein eine besonders wichtige Rolle spielt.

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La Teneur des noyaux en acide désoxyribonucléique (DNA) au cours du développement, de la différenciation et de la croissance

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1. INTRODUCTION

Un des plus grands paradoxes de la cytologie actuelle est de pressentir le rôle fondamental que doit jouer le DNA dans le fonctionnement cellulaire et d'être incapable de l'exprimer de façon concrète.

Comme première approche à l'étude de ce problème, il y avait lieu de rechercher s'il existe des variations éventuelles de la teneur en DNA en fonction de l'activité de la cellule, au cours de son fonctionnement, de sa croissance, ou de sa différenciation.

Cette *étude quantitative* du DNA a été rendue possible, en ces dernières années, par la mise au point de deux ordres de méthodes: (*a*) le dosage chimique sur noyaux isolés; (*b*) le dosage photométrique sur coupes, soit en se basant sur l'absorption des rayons U.V. (méthode de Caspersson), soit sur l'absorption de la lumière visible après réaction de Feulgen.

La comparaison des avantages et des défauts de ces deux méthodes a été faite par Lison (1950*a*, 1953). A vrai dire elles sont complémentaires, l'une (chimique) étant absolue, mais globale, l'autre (photométrique) étant relative mais individuelle. En ce qui concerne la discussion des techniques et leurs causes d'erreurs, cf. Lison (1950*b*), C. Vendrely (1952), et Moses (1952).

2. TISSUS ADULTES, SANS DIVISIONS CELLULAIRES

Lorsqu'il s'agit de tissus adultes qui ne présentent pas normalement de divisions cellulaires (foie, rein, etc.) il semble bien que la *teneur moyenne* présente une grande constance, et tende vers une valeur coïncidant avec deux fois celle du spermatozoïde (Boivin & Vendrely, Vendrely & Vendrely, Ris & Mirsky, Swift, Vendrely, 1952). En général, les données photométriques confirment les résultats dûs aux dosages chimiques, en les tempérant toutefois d'une certaine nuance: les écarts individuels sont notables et ne peuvent être expliqués par des

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raisons uniquement techniques; la fluctuation reste identique après usage de deux méthodes à principe différent tels que la photométrie après réaction de Feulgen ou en lumière ultra-violette à 2570 A° (C. Leuchtenberger, R. Leuchtenberger, C. Vendrely & R. Vendrely, 1952). Il ne s'agit donc pas d'une *constance absolue*, comme l'ont entendu Vendrely & Vendrely ainsi que Ris & Mirsky, mais d'une *tendance vers un certain état d'équilibre* (Lison & Pasteels). L'existence de fluctuations individuelles autour d'une moyenne, implique la possibilité, que dans des cas extrêmes ou accidentels, la moyenne puisse elle-même s'écarter de son taux habituel.

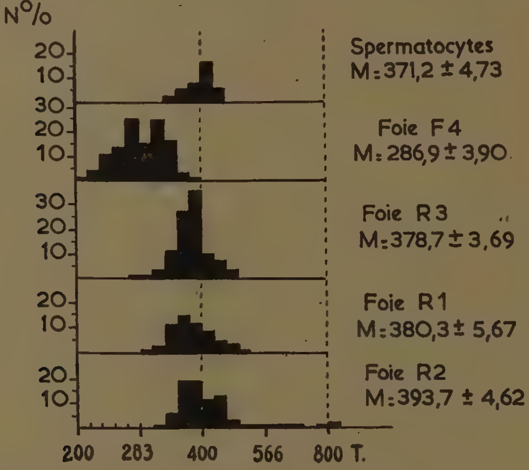

FIG. 1. Histogrammes des teneurs en DNA des foies de quatre rats, comparés aux spermatozoïdes de l'un d'eux. Il s'agit de cellules hépatiques de la classe II (tétraploïdes), tandis que les spermatocytes primaires sont en principe tétraploïdes également. Résultats de Pasteels & Lison: le foie F4 provient de l'animal étudié en 1950 (mesures nouvelles), tandis que les foies R1, R2, R3 proviennent d'animaux nouveaux. En abscisses: teneurs en DNA, calculés sur une échelle logarithmique, des distances égales correspondant à une teneur double. En ordonnées: nombres des noyaux pour chaque catégorie.

La possibilité d'une discordance notable *de ce chiffre moyen* a été démontrée par Pasteels & Lison (1950) dans un cas, vraisemblablement accidentel, mais qui a été confirmé par des mesures récentes (Pasteels & Lison, 1953): cf. Fig. 1. On remarquera sur cette figure, que les histogrammes des foies individuels dont la moyenne est statistiquement identique, ont cependant une allure différente, étant plus ou moins étalés suivant les cas. Ce fait mériterait d'être vérifié par un nombre très considérable de mesures.

3. VARIATIONS EN RAPPORT AVEC L'ACTIVITÉ FONCTIONNELLE

La plupart des résultats, obtenus par la méthode chimique, tendent à montrer qu'il n'existe pas de variation de DNA dans le foie des Mammifères, au cours du jeûne protéique (P. Mandel, M. Jacob, & L. Mandel, 1950; Davidson & Waymouth, 1944; Mirsky & Ris, 1950), ni au cours d'un scorbut expérimental poussé jusqu'à ses dernières limites (Fukuda & Sibatani, 1953). On peut cependant citer trois cas où la méthode photométrique a montré que l'activité de l'organe produisait une altération notable de la teneur en DNA de ses noyaux. Il s'agit du cycle sécrétoire de la glande salivaire de l'Escargot (C. Leuchtenberger & F. Schrader, 1952: variations de 40 à 1); des foies de Rats jeunes et dont la croissance a été arrêtée par un régime aprotéique (Lecomte & De Smul, 1952); du chorion utérin de la femme en fonction du cycle oestral (Vokaer, 1951).

La possibilité de variations de DNA en fonction de l'activité cellulaire est donc démontrée. Des recherches complémentaires sont évidemment nécessaires.[1]

4. VARIATIONS DU DNA AU COURS DU CYCLE MITOTIQUE

Il est un point sur lequel tous les auteurs s'accordent à l'heure actuelle: la teneur en DNA reste constante pendant tout le cours de la mitose; le redoublement du taux de DNA se fait au cours de l'interphase. Il y a toutefois désaccord lorsqu'il y a lieu de préciser le moment précis de l'interphase où ce doublement apparaît.

Comme nous le verrons plus loin, dans des tissus en prolifération, la teneur moyenne s'élève considérablement, et la répartition tend souvent à devenir plurimodale. Pour Swift (1950) et Alfert (1950) cette élévation est due au fait que le doublement de la teneur en DNA apparaît à une période tardive de l'interphase, mais cette interprétation ne repose toutefois pas sur des mesures directes faites à tous les stades du cycle, mais sur un raisonnement indirect.

Des mesures directes faites par Pasteels & Lison à tous les stades de la mitose et au début de l'interphase ont montré que le doublement en DNA apparaît au *début de l'interphase*, au moment de la reconstitution et du gonflement des noyaux. Cette conclusion, qui n'est pas nécessairement générale, s'applique néanmoins à un grand nombre de cas (segmentation de l'Oursin, érythroblastes d'embryons de Rat, glandes de Lieberkühn du Rat adulte, fibroblastes du cœur de Poulet en culture, cellules épidermiques de la souris).

En ce qui concerne les racines de *Vicia faba*, cette conclusion a été confirmée par une méthode d'un principe entièrement différent: l'autoradiographie après incorporation de P^{32} (Pelc & Howard, 1952).

5. VARIATIONS AU COURS DU DÉVELOPPEMENT EMBRYONNAIRE: CROISSANCE OU DIFFÉRENCIATION

De fortes variations de la teneur en DNA, suivant les stades, ont été trouvées par Lison & Pasteels (1951) au cours du développement embryonnaire de l'Oursin *Paracentrotus lividus*. En bref, ces résultats peuvent s'exprimer comme suit: les parties de l'embryon qui sont le siège d'une prolifération active, ou qui *vont* être le siège de cette prolifération, ont une teneur nettement plus élevée en DNA que deux fois celle du spermatozoïde, tandis que les tissus larvaires à faible activité mitotique voient la teneur de leurs noyaux tomber au taux 'normal', deux fois celle du spermatozoïde.

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[1] Note au moment de la correction des épreuves: un quatrième cas a eté signalé depuis, cf. Bergerord & Tuchmann-Duplessis (1953), *C.R. Acad. Sci. Paris*, **256**, 1080.

Moore (1952) a observé de même des variations de la teneur en DNA au cours du développement de *Rana pipiens*. D'après cet auteur cependant, ces variations de la teneur en DNA ne seraient pas corrélatives à la croissance des tissus embryonnaires, mais plutôt à leur degré de différenciation. Mais il semble toutefois que Moore ait attribué trop de crédit à l'index mitotique, qui, lorsqu'on ignore le rapport chronologique entre l'interphase et la mitose, ne permet aucune interprétation. Il n'est pas possible actuellement de démontrer que, en dehors du taux de prolifération mitotique, la teneur en DNA serait aussi liée au degré de différenciation cellulaire.

6. TENEUR EN DNA DANS LES TISSUS ADULTES EN CROISSANCE

D'après Lison & Pasteels, la teneur élevée des noyaux dans les tissus en prolifération n'est donc pas due à une augmentation du DNA à un stade tardif de l'interphase (cf. plus haut); elle doit donc être considérée comme une *caractéristique générale* du tissu en prolifération, *tant au repos qu'en métaphase*. Ceci peut être démontré par le tableau 1 où sont exprimées les valeurs trouvées dans divers tissus en prolifération, soit au cours de l'interphase, soit au cours de la métaphase, en prenant comme unité la teneur du spermatozoïde correspondant.

TABLEAU 1

	Tissus en prolifération		*Références*
	Interphase	*Métaphase*	
Glande Lieberkühn Rat	2·4	2·4	(Lison & Pasteels, 1950 et obs. inéd.)
Erythroblastes embryon Rat. 9 j	3·4	3·4	(Lison & Pasteels, 1950)
Erythroblastes sang circulant Pleurodèles:			
T 3	3·6	?	(Pasteels: obs. inéd.)
T 2	2·3 à 5·6	4·8	
1e division segmentation Oursin	5	4·5	(Lison & Pasteels, 1951)
3e division segmentation Oursin	5·2	5	
	Tissus au repos		
Spermatozoïdes	1	—	
Tissus adultes divers	±2	—	(Nombreux auteurs)

Nous remarquerons donc (*a*) que la teneur en DNA des tissus en prolifération est plus élevée que celle des tissus en repos; (*b*) qu'elle est élevée tant au cours de l'interphase que de la métaphase; (*c*) qu'elle varie d'un tissu à l'autre; (*d*) qu'elle ne présente pas un rapport simple par rapport à la teneur du spermatozoïde.

Nous terminerons cette démonstration par des données inédites démontrant que *dans un même tissu,*[1] *on peut faire varier le taux de DNA en fonction de la croissance.*

On sait que le sang circulant des Batraciens contient des érythroblastes immatures, et éventuellement des mitoses. Cette prolifération des cellules sanguines varie d'un animal à l'autre, suivant leur état physiologique (nutrition, facteurs saisonniers, etc.). La Fig. 2 montre les histogrammes comparatifs résultant de mesures faites sur les frottis de deux Pleurodèles (T 2 et T 3). On remarquera que la teneur moyenne des érythrocytes dans les deux cas est identique, ainsi que celle des polynucléaires de l'animal T 2 (non mesurés chez T 3). Ces deux animaux différaient par le nombre de cellules immatures, beaucoup plus nombreuses chez T 3. Or, l'histogramme des teneurs en DNA des cellules immatures est à la fois plus étalé et plus élevé pour T 2 que pour T 3 (cf. aussi Tableau 1).

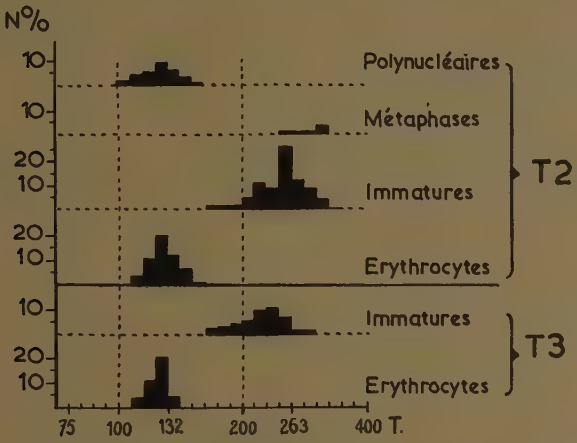

FIG. 2. Histogrammes de teneurs en DNA de diverses cellules sanguines de *Pleurodeles waltii.* Comparaison de deux animaux. Résultats inédits de Pasteels. Mêmes abscisses et ordonnées que pour la Fig. 1.

Le second exemple sera tiré d'un travail en cours, en collaboration avec W. S. Bullough. Une souris a eu l'oreille droite frottée à l'huile de croton, l'oreille gauche servant de témoin, ce qui a produit une différence très accentuée dans le taux des mitoses. Des fragments de ces deux oreilles ont été fixés simultanément. Une semaine plus tard, l'animal a été injecté à la colchicine, puis 5 h. après cette injection, deux nouveaux fragments ont été fixés. Aucune différence n'a pu être constatée entre la teneur en DNA de l'épiderme d'une oreille, avant, ou après l'injection de colchicine. Mais, à la suite de cette injection de colchicine, nous avons été à même de mesurer un nombre considérable de *métaphases* dans l'oreille gauche (témoin) et dans l'oreille droite (traitée à l'huile de Croton). Voici les résultats (Tableau 2).

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[1] Les erreurs techniques dues à des distributions dans des noyaux à structure différente peuvent donc être exclues.

TABLEAU 2

Teneurs en DNA des métaphases dans l'épiderme d'une oreille de Souris

	Nombre de mesures	*Moyenne*
Oreille gauche (Témoin)	75	401·36±8·19
Oreille droite (Huile de Croton)	83	530·30±8·60

La différence est hautement significative.

Conclusion: *lorsque la fréquence des divisions cellulaires d'un tissu est augmentée, la teneur en DNA des métaphases est corrélativement augmentée.* La même conclusion vaut évidemment pour l'interphase.

RÉSUMÉ

1. Dans des tissus adultes, au repos, on constate généralement une constance de la teneur moyenne en DNA, équivalent à deux fois celle du spermatozoïde.
2. Cette constante de la moyenne n'exclut pas des fluctuations autour de la moyenne.
3. Des écarts notables de cette moyenne ont pu être constatés, soit de façon accidentelle, soit en liaison avec l'activité cellulaire.
4. Le doublement du DNA au cours du cycle mitotique se fait de toute façon au cours de l'interphase; des mesures directes faites par Pasteels & Lison montrent que, dans de nombreux cas, ce doublement apparaît au début de l'interphase.
5. Au cours du développement embryonnaire, il a été possible de montrer une augmentation du taux de DNA en liaison avec la croissance; mais on n'a pas pu démontrer jusqu'ici une liaison nette avec la différenciation cellulaire.
6. Une augmentation qui ne se fait pas nécessairement suivant des multiples de simple à double apparaît dans les tissus adultes et en prolifération. Elle s'observe autant au cours de la métaphase que de l'interphase; elle peut être modifiée expérimentalement en fonction du taux de croissance.

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The Formation of Haemoglobin during the Development of the Erythrocytes

by BO THORELL[1]

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FROM the functional point of view the processes of differentiation during the formation of the red blood-cells are characterized by the synthesis of haemoglobin.

If the haemoglobin content of single erythroblasts is measured one finds that most of the formation of haemoglobin occurs in the later stages of erythropoiesis (Table 1).

TABLE 1

Cell diameter, μ		*Total amount of* Hb (10^{-12} *g.*)
Growing erythroblasts	11·0	0
	10·5	0
	9·5	0·5
	8·5	2
	8·0	4
	7·5	10
	7·0	22
	6·5	25
	Mature cell	28

When it reaches these stages of development the growth and division processes of the erythroblasts have been completed. The cytoplasm has lost its basophilia and the nuclei are becoming pycnotic. The cells are, however, capable of forming haemoglobin.

From the general point of view of cell physiology, it seems reasonable to believe that only the prosthetic group (haem) is being synthesized during these later stages of erythropoiesis. The globin-protein might be manufactured mainly in earlier, growing erythroblasts in the presence of ribosepolynucleotides which are abundant in the cytoplasm of these cells.

To test this view, a series of experiments was made in collaboration with Dr. Hammarsten, using hens which were regenerating red cells intensely after a

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single, severe haemolysis produced by an injection of phenylhydrazine. Thirty-six hours after the haemolysis the content of immature erythroblasts in the peripheral blood exhibited a sharp rise. The peak value was reached around 60 hours, after which time the cells in the blood were undergoing their final maturation (Fig. 1).

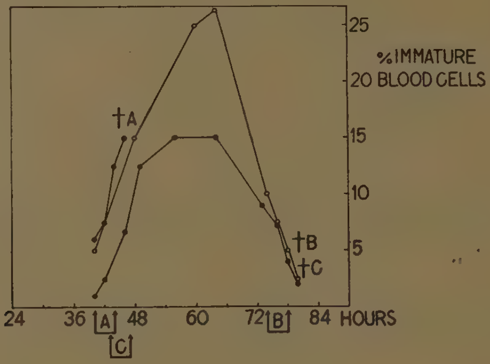

FIG. 1. Curves representing the proportion of immature cells, containing less than $5 \cdot 10^{-12}$ g. of haemoglobin, in the peripheral blood of the experimental animals. The short curve on the left is for hen *A*; the lower curve for hen *B*; and the upper curve (points marked with open circles) for hen *C*. On the abscissa is plotted the time after phenylhydrazine haemolysis. The arrows indicate the first and last injection of labelled glycine in each hen.

At different stages of this blood-cell regeneration the hens were given glycine labelled with N^{15} intraperitoneally. Glycine was chosen because it is directly incorporated into the haemin and the proteins (Shemin & Rittenberg, 1946; Eliasson *et al.*, 1951). After a certain lapse of time the hens were sacrificed by exsanguination, and the different substances in the blood-cells were separated, degraded, and analysed.

Thus we aimed at investigating whether or not the ratio of N^{15} incorporation into the globin and into the haemin was the same in erythroblasts at different stages of maturation.

Fig. 1 shows the principle of such an experiment. The first hen (*A*) was given isotope during a period when the amount of immature cells in the peripheral blood was increasing. Six hours later the animal was sacrificed and the isotope concentration in the haemin and the globin analysed. The second hen (*B*) was given labelled glycine when the immature blood-cells were decreasing in amount, i.e. during their final maturation. It also was killed 6 hours later. The third hen

(*C*) received isotope during a period when its immature blood-cells were increasing, but it was not sacrificed until 38 hours later when the blood-cells were in the process of final maturation.

It will be seen in Table 2 that in the first hen globin synthesis predominates in relation to haemin synthesis. In the second hen the condition is reversed; haemin production predominates in the blood-cells undergoing their final maturation. The third hen shows a markedly dominant incorporation in the globin, indicating an intense globin synthesis in the cells during the period around the isotope injection. The level of N^{15}-concentration in glycine at the time of the haemin synthesis has been low.

TABLE 2

	Atom % excess N^{15} *in glycine isolated from globin*	*Atom % excess* N^{15} *in haemin*	*Ratio haemin/glycine*
A	1·627	1·177	0·72
B	0·956	1·558	1·63
C	5·29	0·465	0·09

Thus the ratio of the incorporation values for globin to those for haemin isolated from erythroblasts at different stages of maturation indicates that during erythropoiesis the maximum rate of the synthesis of globin precedes that of haemin.

In general terms it seems reasonable to suggest that the endocellular synthetic processes which form the basis of the functional differentiation of the erythroblasts are separated and distributed over a relatively wide range of the development. During a certain phase of this development the cell has a given pattern of enzymatic activities and a given part of its functional differentiation is elaborated.

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Growth as related to the Cell Cycle in Single-cell Cultures of *Tetrahymena piriformis*[1]

by ERIK ZEUTHEN[2]

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INTRODUCTION

FOR single-cell cultures of *Paramecium* grown in capillaries, Giese (1945) found exponential multiplication to a 32–64-cell stage. This inspired the hope in the writer that synchronism of divisions in single-cell cultures of *Tetrahymena* might be maintained over a number of cell cycles, so that accurately recorded growth curves for such cultures would supply information about the activity of the growing cell in relation to the cell cycle.

The experiments were performed with pure cultures of the ciliate *Tetrahymena piriformis*, Lwoff's strain (experiments 1, 2, and 4) or Kidder's strain W (experiment 3). For each experiment 1 cell, taken from a 2–4 days' old culture (which had passed beyond the exponential growth phase), was grown bacteria-free in a microrespirometer on a small amount (0·05–0·155 μl.) of nutrient medium (2 per cent. proteose-peptone (Difco) + 1 per mille liver fraction L (the Wilson Laboratories) in tap-water).

By convention, the rate of respiration was taken as a measure of the amount of respiring material. The resulting curves ($O_2/\text{min.} = V_t$, plotted against hours) are therefore to be dealt with as growth curves, and the slopes of the curves indicate rate of synthesis. All conclusions derived are preliminary; they should in due time be checked, with more experiments and also other measures of growth.

METHOD

Space prevents a detailed description of the method (see Fig. 1, I and II) used for respiration measurements. It will be published in *Experimental Cell Research*. It combines the breaking pipette technique of handling single cells in small volumes (Holter, 1943) with the use of the broken-off tip of the pipette as a Cartesian diver respirometer. Fig. 1, I will give some idea of the way the diver is made and charged. Sterile conditions are extremely easy to maintain. The diver's equilibrium pressure is adjusted and measured using a new 'sensitive manometer'

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[1] Formerly *Colpidium piriformis* or *Tetrahymena geleii.*

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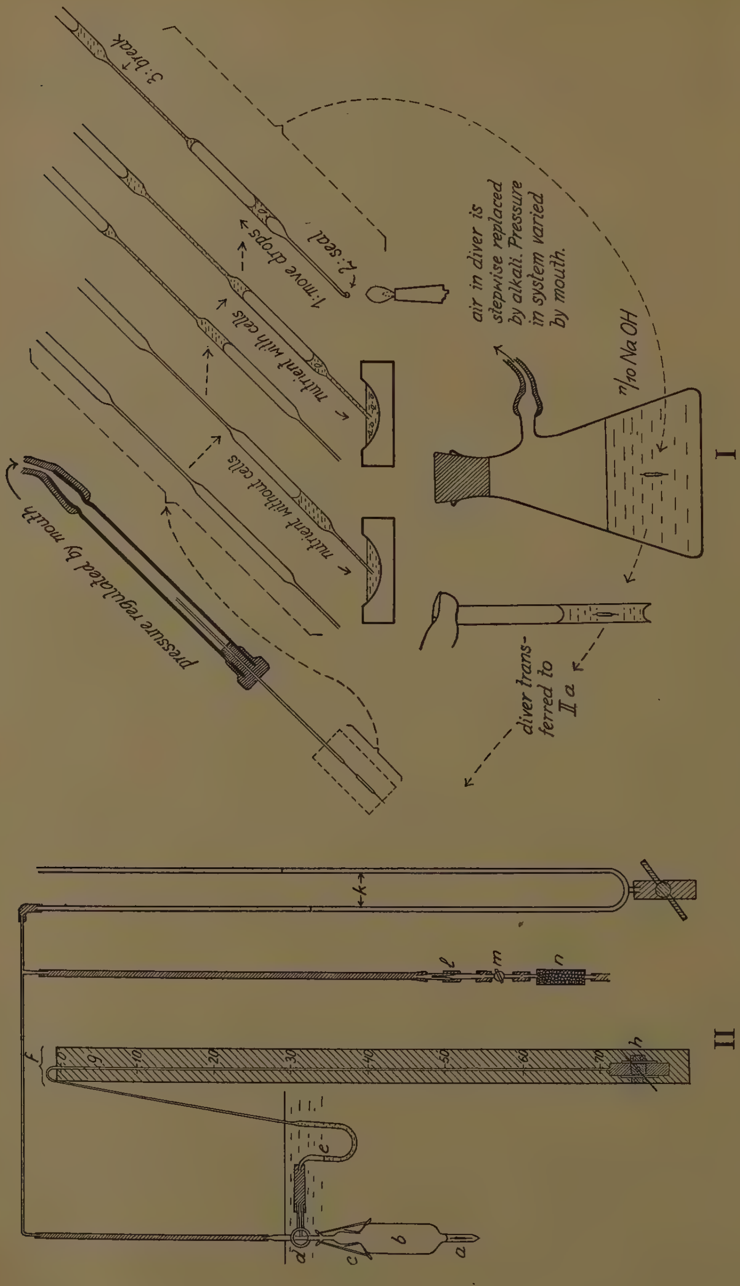
3: break
2: seal
1: move drops
nutrient with cells
nutrient without cells
pressure regulated by mouth
air in diver is stepwise replaced by alkali. Pressure in system varied by mouth.
n/10 Na OH
diver transferred to II a
I
a
b
c
d
e
f
0
5
10
20
30
40
50
60
70
h
l
m
n
k
II

FIG. 1

FIG. 1, I. The tip of a glass capillary is pulled in a microflame to form what is to be the diver respirometer. The pointed tip should be about 5–7 mm. long, the wider piece about 1 cm., and the narrow channel about 1 cm. Inner dimensions of the order 50 μ, 500 μ, and 50 μ, respectively. To select the right capillary from which to make the diver one should aim at a tube which will float in N/10 NaOH when filled with air to about half its length. After transfer to the vacuum bottle shown below in the figure the diver is made buoyant. Suction is applied so that first the nutrient medium filling the narrow channel, then air, is sucked out of the diver. When normal pressure is re-established, air has become replaced by alkali. This is repeated in steps so that buoyancy is approximated.

FIG. 1, II. The diver floats in N/10 NaOH in *a*, thus freely exposed to the pressure prevailing in the air-space *b* which extends through *c* and *d* to the water surface *e*. At the beginning of the experiment the pressure in *b* is adjusted by mouth through *n*, *m*, *l*, with *d* in the position shown in the figure. With *m* closed, the manometer *k* is useful for the fine regulation of the initial flotation pressure in *b*. *n* is a CO_2-trap, *l* is an air-brake.

During actual measurements *d* is closed to *l*, *m*, *n* and to *k*, but open to *e*.

The volume of the 'burette' from 0 cm. to 70 cm. is less than 2 % of the volume of *b*, extending to *e*. Therefore linear movements of *g* in the (very uniform) 'burette' sets up proportional (within 2 %) pressure changes in *b*. The system can be reset when *g* has been moved across the scale. The gas exchange per unit time $\left(\frac{\Delta O_2}{\Delta t}\right)$ is calculated from the formula

$$\frac{\Delta O_2}{\Delta t} = \frac{x.v.g_D.f(B+h-e).273}{(V+v).10300.(273+t^0)}$$

in which x is the movement (in mm. per unit time) of the bubble g in the 'burette', v is the volume (μl.) per mm. 'burette', V is the volume of b extending to c (*c.* 100,000 μl.; $\frac{v}{V} = 2.10^{-5}$), g_D is the dry weight (mg.) of the diver determined after the experiment. f is a factor—determined independently for the glass from which the diver is made—indicating the ratio between volume air (μl.) and dry weight (mg.) of glass when the system is buoyant. B is the barometric pressure, mm. H_2O (760 mm. Hg $\sim$ 10300 mm. H_2O). h is the initial equilibrium pressure of diver read on k as the difference in height of the menisci (mm.). e is the vapour tension of water (or of N/10 NaOH and of 2 % proteose-peptone) at t^0.

In the present studies h in the equation has been small compared to B. It should be noticed, however, as an interesting and promising feature of the present method, that x varies inversely with $(B+h-e)$. Therefore the sensitivity of the system should increase greatly at low pressures and should approach infinity when h approaches negative values equal to $B-e$.

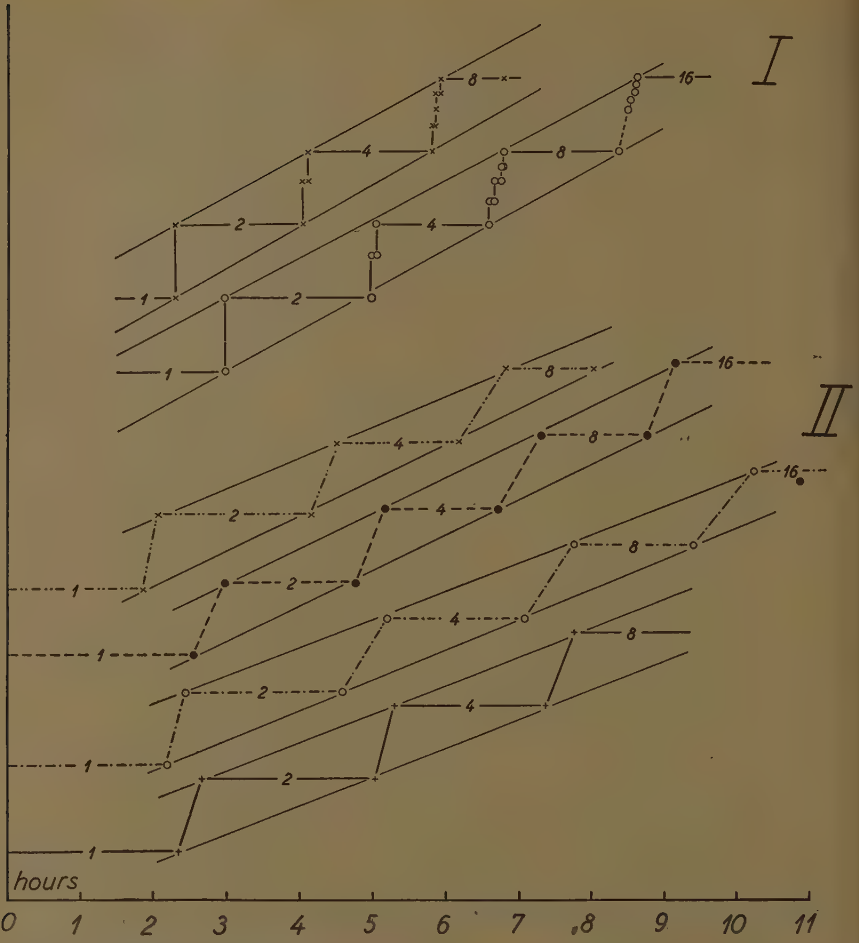

FIG. 2. In single-cell cultures the periodicity of cell division is maintained over a number of generations. Logarithmic ordinate scales omitted, because each experiment requires its own scale. Each step on curves corresponds to a doubling in numbers. Generation times (g.t.): for I: 108 and 113; for II: 133, 122, 152, and 152 min.

(Fig. 1, II) which is really not a manometer at all but a burette (II, *e*, *f*, *g*, *h*) by means of which accurately measured volumes of fluid can be withdrawn from a closed air space (II, *b*) in which regulated pressure changes are thereby created. The amount withdrawn is read by the movements of the air bubble *g*. The diver floats in a small amount of alkaline medium in a pocket (II, *a*), open to the air space. The 'manometer' can be adjusted to the nearest 1/25 mm. water pressure $\sim 4.10^{-6}$ atm. The diver is sensitive to around this limit, and since its gas volume is 0·5–1 μl. the sensitivity of the method is of the order 2–4 . 10^{-6} μl. O_2 uptakes were measured over about 10-minute periods, but were plotted for 1-minute periods in the middle of the interval for which they were measured. The high sensitivity and the high ratio (order 10^5): oxygen capacity / sensitivity of this diver makes it excellently suited for long-continued growth studies. For further technical information, see the legend to Fig. 1, I and II.

RESULTS

The experimental results are shown in Figs. 2, 3, and 4 and in Table 1. In Figs. 2 and 4 the ordinate scale is logarithmic, in Fig. 3 it is equidistant. In Fig. 2 the upper set of curves (I) are control experiments (by Mr. H. Thormar) on single cells multiplying in capillaries, not themselves used as respirometers; the same nutrient medium was used as in the respiration experiments, but made up with double glass distilled water; salt mixture as in Kidder's medium A (Kidder & Dewey, 1951). Cell numbers were reckoned to increase by one each time two daughter cells were observed to separate, and in the two experiments all divisions were very carefully observed (except for a short period of time in the final part

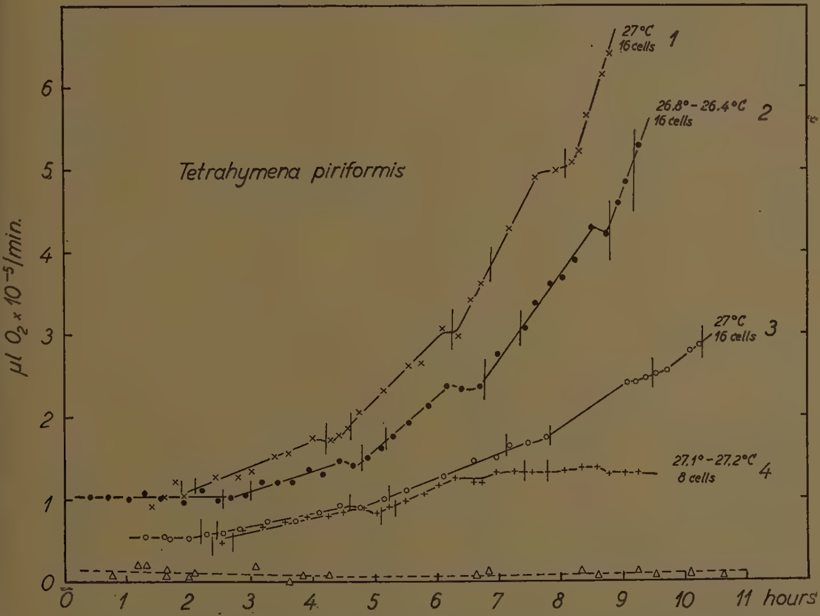

FIG. 3. Rate of respiration plotted on an equidistant scale against hours. Of each pair of vertical lines intersecting the curves, the first one indicates onset of division activity, the second indicates that the daughter cells have separated. All experiments start with 1 cell; the number of cells recovered at the end of the experiment is indicated.

of the one experiment, curve dotted). In the graph, log cell numbers are plotted against hours of development at 27°. Each step on the curves corresponds to the logarithm of 2 and thus to a doubling of cell numbers.

The next following set of curves (II, Fig. 2) indicates how cell numbers were observed to increase in the four respiration experiments, shown in the curves 1–4, Figs. 3 and 4. In this case, with each new division, cell numbers were assumed to double over the period from the time the first cell was observed to begin furrowing till the last pair of division cells had separated in the diver. By the end of the third or the fourth cycle this assumption was checked by actual cell counts; and the expected number of cells (8 or 16) was obtained.

TABLE 1

Single Cells of Tetrahymena piriformis, *multiplying on Proteose-Peptone + Liver Extract in Tap-water*

Characteristics of Growth Curves 1, 2, and 3, FIG. 3, *after Correction for the Blanks*

Number of cells	*Rate of growth* (Δ*v*/*hour*) *calculated from slopes in the figure*		
	Exp. 1	*Exp.* 2	*Exp.* 3
I 2–4*	3·0	2·5	1·6
4–8	7·5	6·2	3·3
8–16	14·5	10·5	5·0
16	24·0	20·5	not defined
	Extent of growth (Δ*v*/*generation*) *as measured from one plateau to the next*		
II 2–4*	7·0	4·0	4·0
4–8	13·0	8·5	7·0
8–16	20·0	19·0	8·0
	O_2/*min.* (*v*) *consumed in plateau, prior to fission*		
III 1	9·5	9·5	4·5
2	16·5	13·5	8·5
4	29·5	22·5	15·5
8	49·5	41·5	24·0
	Same, per cell		
IV 1	9·5	9·5	4·5
2	8·3	6·8	4·3
4	7·4	5·6	4·1
8	6·2	5·2	3·0
Diver, vol. μl. . . .	0·89	1·06	0·63
Nutrient medium, μl. . .	0·111	0·155	—
Cells by end of exp. . .	16	16	8
Cells/ml. after exp. . .	144,000†	103,000	—

$v = \mu l.O_2.10^{-6}$/min. Δv = increase in v per stated period of time.

* 2–4 means: from beginning of furrowing at the end of the 1-cell stage and through most of the 2-cell stage (Table 1, I), or till the time when the two cells begin to furrow prior to division into 4 (Table 1, II). Analogous meaning for 4–8 and 8–16.

† Next day, while still in diver, 450,000.

For each experiment (I, II, Fig. 2) two straight lines were made to fit the upper and the lower 'edges' of the stepwise curves. The mean of the slopes for each set of two lines gives the generation time, g.t., which was found to vary from 108 to 152 minutes in the experiments reported. The straight-line fit indicates that the data are interpreted to show that cellular multiplication was approximately exponential in all cases.

The first method of following cell multiplication is based on the observation of a definite morphological event, which in the single cell occurs infinitely fast. The second method is based on the observation of the complete cytoplasmic fission, in each cell occupying about 8 per cent. of the cycle. As expected, the resulting curves show more abrupt jumps with the first than with the second method. All curves are interpreted to show that, starting with a single cell, a rather nice periodicity of divisions is maintained over at least 3–4 generations.

Fig. 3 shows the four respiration experiments (1–4) and also two experiments (both given triangular symbols) with divers filled as usual, but without *Tetrahymena* cells. They serve as blanks and also as controls of sterility. With the possible exception of curve 4 (not included in Table 1, because the scattering is of the same order as some of the phenomena to be observed), all experiments seem to indicate respiratory periodicity correlating with the cell cycles.

DISCUSSION

1. With reference to Fig. 3, the first point to be stressed is that in most cases the curves can be interpreted as showing that the rate of respiration increases linearly with time, usually beginning with the onset of the cytoplasmic fission (periods of fission indicated by frames), continuing through the process of fission, and ending 15–30 minutes prior to the onset of the next fission. This observation of constant rates of synthesis between divisions should be checked by more experiments. As the results stand, they suggest that although we deal with microbial cultures in their exponential phase of growth, synthesis between divisions is not an exponential function of mass, but rather a simple function of the number of individuals present. Therefore, growth between divisions does not—as might be inferred from the words logarithmic or exponential—seem to be an autocatalytic event.

2. Again with reference to Fig. 3, the next observation to be stressed is a tendency (for an exception see curve 3) for the rate of synthesis to double just prior to the onset of the cytoplasmic fission. In other words, although the rate of synthesis appears to be governed by the number of cellular units, the important thing may not be the number of cells *per se*, but rather the number, in the cells, of undefined centres doubling before each cytoplasmic fission. Such centres governing the rate of synthesis could be the macronucleus or it could be other cellular organelles. *Tetrahymena piriformis* is known not to contain micronuclei and the macronucleus is known to divide amitocally, prior to and during the

cytoplasmic division (Furgason, 1940). Prior to amitosis ‘reorganization bands’ have been observed to move across the macronucleus.

3. Fig. 3 indicates a third point which should be stressed, that prior to doubling of the rate of synthesis there seems to be a respiratory plateau, or maybe a slight dip, lasting 15–30 minutes. Whatever the ‘synthetic centres’ are, they seem to have the alternative of duplicating themselves (in the respiratory plateau) or of controlling synthetic processes in the whole cell.

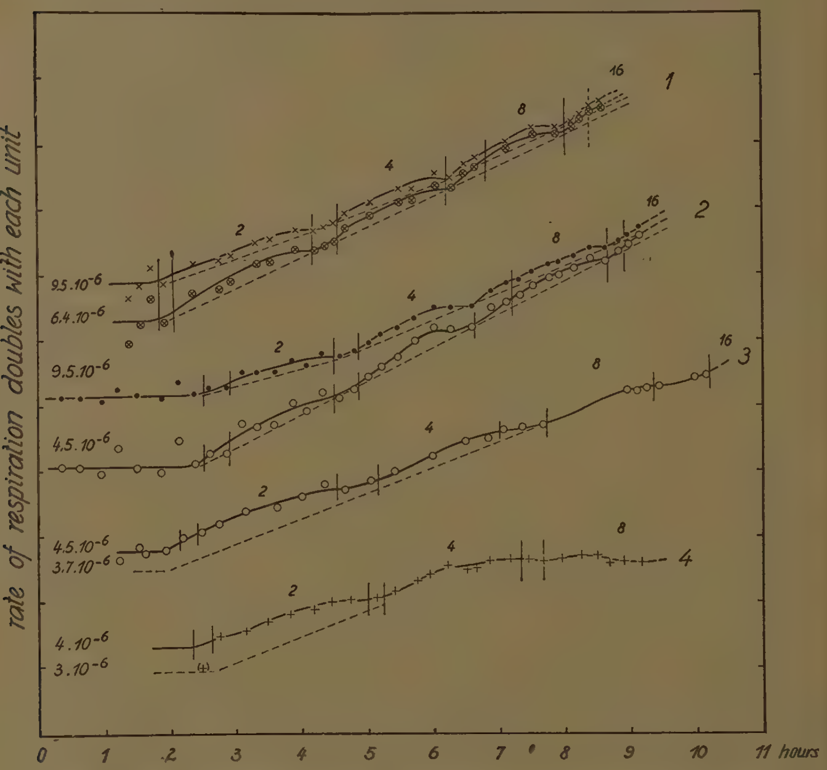

FIG. 4. Same experiments as in Fig. 3, plotted as logarithms against time. See text.

4. With special reference to Fig. 4, the overall shape of the growth curves should be discussed.

When plotting the logarithms of the total respiratory rate ($v_t = O_2/\text{min.}$) against hours of growth the four curves result which in Fig. 4 are designated $9{\cdot}5 . 10^{-6}$ (experiment 1), $9{\cdot}5 . 10^{-6}$ (experiment 2), $4{\cdot}5 . 10^{-6}$ (experiment 3), and $4 . 10^{-6}$ (experiment 4). Thus the curves are labelled according to the rate of oxygen uptake (μl. O_2/min.) which the initial cell showed before growth began in the respirometer (see Table 1). The position of all curves on the logarithmic ordinate scale is arbitrary; we are concerned only with the slopes.

In experiment 2, perhaps also in experiment 3, there is a definite lag phase before growth is resumed on the fresh medium, supplied with the introduction of the cell into the diver. It may be significant that this lag (in experiment 2) is broken by a cell division, because prior to each new division—even in the fast multiplying culture—there is a short lag, the respiratory plateau. Whether justified or not, for the initial parts of the other experiments (1, 3, and 4) the curves have been drawn in accordance with the findings in experiment 2.

Inspecting, throughout their course, the two curves which are both designated by $9{\cdot}5 \, . \, 10^{-6}$, the respiratory periodicity shows up rather clearly. Disregarding the periodicity, however, it is apparent that the overall trend of both curves deviates from linearity. In other words, although multiplication is logarithmic (Fig. 2), synthesis, even in its overall course, does not appear to be so.

However, in their main trend the two curves can be transformed into straight-line logarithmic curves if we accept that not the total initial respiratory rate (v_0) but only part of it ($v_0 - a$) shows logarithmic growth with a doubling time equal to g.t.

The two experiments can thus be described by the formula

$$\log (v - a) = k \, . \, t. \qquad (1)$$

In experiments 1 and 2 ($v_0 - a$) approximates $6{\cdot}4 \, . \, 10^{-6}$, and $4{\cdot}5 \, . \, 10^{-6}$ (μl. O_2/min.) respectively; and the non-growing constant fraction (a) seems to represent nearly one-third and one-half respectively of the total initial rate. The characteristics of the curves representing experiments 1, 2, and 3 are given in Table 1. Section I of that table gives the slopes of the straight lines between divisions (Fig. 3) and demonstrates the basis on which a doubling in rate with each division has been suggested (paragraph 1 of discussion). Section II of the table demonstrates the total gain, Δv, in respiratory rate per generation. Except for the last period in experiment 3, Δv is also accepted as showing doubling with each new generation. Sections III and IV show that—due to the existence of the non-growing fraction a—the cells get smaller and smaller with each generation, as measured by the amount of O_2 they consume per unit time. In experiments 1 and 2, and in agreement with Ormsbee (1942), this was checked by the visual observation that the cells get progressively smaller in the first generations.

Experiments 3 and 4, so far omitted from this discussion, leave us in doubt whether in these two experiments the volume of nutrient medium (not measured, but in both cases smaller than in experiments 1 and 2) ever permitted unrestricted growth to take place, that is, whether ($v_t - a$) ever grew exponentially with time. In experiment 3 (section II, Table 1) Δv/generation doubled from the first generation to the next, but remained nearly constant from the second to the third. In the same experiment the rate of synthesis (Δv/hour, section I, Table 1) nearly doubled with each generation. The slope over the third generation may have been slightly lower than that corresponding to a doubling from the previous generation, although hardly significantly so. This situation suggests that when growth becomes limited by the volume of nutrient medium available, the first thing to happen is that the respiratory plateau (paragraph 3 of discussion) increases in duration at the expense of the preceding period of straight-line increase in respiring mass. Experiment 4 suggests that this develops to a point where the respiratory rate fluctuates up and down in broad waves, with little overall increase, resembling the situation prevailing in dividing eggs (Zeuthen, 1950 *a*, *b*, reviewed in 1949, 1951; Scholander *et al.*, 1952).

The uncertainties mentioned, greater for experiment 4 than for experiment 3, prevent the determination of the value of *a* in equation (1) in these two experiments. However, in Fig. 4 the dotted lines below the two curves representing experiments 3 and 4 indicate which overall slope in each case would have corresponded to a doubling in mass over the first generation. This slope is higher than the one found for the total respiration. Hence the cell with which the diver was first charged in experiments 3 and 4 may not have been capable of growing with its whole body. By analogy with the conclusions derived from experiments 1 and 2 the curves for experiments 3 and 4 will give some idea of the possible value of *a* also in experiments 3 and 4.

5. *Tetrahymena* cells grown in mass culture from stationary phase cells are known in the early phase of growth progressively to reduce to about 60 per cent. of the initial weight and again, after the end of the exponential phase, to grow bigger. Ultimately, big stationary phase cells can be recovered (Ormsbee, 1942). Late in the stationary phase cells differentiate, e.g. they become highly polymorphic with respect to shape and size of cell body and cell nucleus.

In the four respiration experiments reported the initial level of respiration varied from cell to cell, although for three of them (curves 1, 2, and 4) cells were from the same clone. As has been demonstrated, the progeny of each cell retains constant growth characteristics (see curves, Fig. 3 and 4, and Table 1), probably till the end of the exponential phase. Thus, with respect to growth capacities, the individuality of the initial cell was transmitted to several subsequent generations. Accordingly the present results, together with those of Ormsbee, may indicate that in *Tetrahymena* differentiation is limited to post-exponential phases of growth. The outcome of this fifth point to be discussed is that one aspect of differentiation in stationary phase cultures is that cells may grow too big for the synthetic machinery which they possess and which becomes reactivated, after a lag, upon transfer to fresh nutrient medium.

The comparison made above between a dividing egg and a multiplying ciliate may turn out to be quite superficial. Nevertheless, this presentation has served the purpose of demonstrating, on a less complex system, which factors may be involved in producing respiratory rhythmicity in the cleaving egg. While the egg represents a closed system, *Tetrahymena* may grow on fully defined media, as demonstrated by Kidder and collaborators (1951) and by Elliot (1950, 1951), with all the possibilities for controlling limiting factors which lie therein. It is planned to extend the present studies to cells growing on defined media, but so far we have met difficulties because on such media there has been a low degree of synchronism of divisions.

SUMMARY

1. There is some evidence that synthesis in single cells of *Tetrahymena* occurs with very nearly constant rate when traced from one division to the next.
2. Overall exponential growth over a number of cell cycles may be the statistical result of the fact that centres—of which each one is in control of synthetic processes occurring with constant rates—multiply by discrete doublings, i.e. exponentially, in close relation with the process of cell division.
3. While this doubling occurs there may be a provisional block to further increase in respiratory rate.
4. In the post-exponential growth-phase cells may differentiate and some may grow too big for their synthetic machinery, as—after a lag—it becomes reactivated upon transfer to fresh nutrient medium. For this reason overall exponential growth over a number of cell cycles may pertain only to a fraction of the cell with which the experiment was started.

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Sea-urchin Development in the Light of Enzymic and Mitochondrial Studies

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THE sea-urchin egg is characterized by a very high morphogenetic plasticity. Its trend in differentiation can in fact be controlled by means of various chemical agents. The development of the entoderm is thus favoured at the expense of that of the ectoderm by adding lithium ions to the sea-water (Herbst, 1892; Lindahl, 1936). Iodosobenzoic acid (Runnström & Kriszat, 1952) and thiocyanate (Herbst, 1892; Lindahl, 1936) have an opposite effect, i.e. they favour ectodermal development. The mechanism of segregation of the egg into the primary germ-layers might be elucidated if we knew more about the biochemical mode of action of these agents. A series of biochemical studies on lithium-treated and normal eggs and larva was therefore undertaken. The studies began on the level of amino acids and peptides and continued on the levels of enzymes and intracellular inclusions.

The total changes in the amino-acid composition of the egg were found to be rather small (Gustafson & Hjelte, 1951). Changes generally did not appear until the hatching stage or around the onset of visible differentiation (mesenchyme-blastula stage). They were most pronounced in the fraction of free amino acids and peptides, where changes already occurred during cleavage stages. The appearance of new antigens could not be demonstrated before an early gastrula stage (Perlmann & Gustafson, 1948). Between this and the 48-hour stage a new component appeared.

Studies on enzymic development reinforced the impression that the cleavage period is a 'silent phase' (Augustinsson & Gustafson, 1949; Gustafson & Hasselberg, 1950, 1951; Mazia *et al.*, 1948). Thus the activity of a series of enzymes was low and rather constant during this period, but increased in the late blastula stage or during gastrulation (alkaline phosphatase, dehydrogenases, glutaminase I, cathepsin II, apyrase, and cholinesterase). In another series of enzymes the activity was constant throughout early development up to the pluteus stage (aldolase, adenosinedeaminase, a proteolytic enzyme, phenolsulphatase, enzymes splitting inorganic pyro- and hexametaphosphate (Gustafson & Hasselberg, 1951) and a peptidase (Holter, 1936)).

Several of the enzymes with increasing activity are to a considerable extent localized in mitochondria, whereas those enzymes showing a constant activity as

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a rule are non-mitochondrial. The percentage rise in activity was, however, not uniform. There was also some variation with respect to the time of the rise in activity. We believe, however, that this rise mainly reflects an increase in mitochondrial number at the onset of visible differentiation (Gustafson & Hasselberg, 1951). Li treatment during cleavage stages retarded the subsequent rise in activity of a series of enzymes, and also retarded changes in the amino-acid composition. Li treatment during cleavage stages should thus check the subsequent rise in mitochondrial number.

The above hypothesis was supported by mitochondrial counts (Gustafson & Lenicque, 1952). Particles stainable with the mitochondrial vital stain Janus green were observed in the pluteus. These bodies were also stainable with nile blue sulphate and dahlia violet. They were also recognized in the phase microscope as spheres or dumbbell-shaped structures. After nile blue staining the particles resembled granules or rods, as a rule with thickened ends. The stainability may be due to a rim of basophilic material, which often surrounds the mitochondria (Opie, 1947). The nile blue stainable elements were counted *in situ* in larvae flattened beneath the cover-slip. Curves of the relative mitochondrial number in different early stages were obtained by using the method described above. These curves resemble the enzyme curves, even with regard to the retarding effect of lithium treatment (Gustafson & Lenicque, 1952).

The cytological and the enzymological picture of early development are evidently in good agreement. There are, however, exceptions, such as cytochrome oxidase. This enzyme shows a drop in activity in homogenates at stages preceding the rise in mitochondrial number (Deutsch & Gustafson, 1952). A similar case has been reported from amphibian development (Spiegelman & Steinbach, 1945). In both cases observations with different methods strongly indicate a synthesis of the enzyme (Boell, 1947; Lindahl, 1939). The drop must then reflect some condition obscuring the enzyme synthesis, such as a gradual aggregation of enzymic elements to mitochondria or the development of a surrounding membrane. Another possibility is that the drop is brought about by an inhibitor which depresses the activity of iron-porphyrin-enzymes (Hargreaves & Deutsch, 1952). Such an inhibitor has been demonstrated in homogenized sea-urchin material, where it is at least partly responsible for the drop in catalase activity in late blastula stages (Deutsch & Gustafson, 1952). Analogously, the formation of glutathione or some other enzyme activator may cause activity changes which do not correspond to enzyme syntheses.

The rise in alkaline phosphatase activity is not checked by lithium treatment as the activity of mitochondrial enzymes seem to be. This indicates that the major part of the enzyme has a different localization, e.g. in the cell membranes (cf. Danielli, 1952), in microsomes, or in the nuclei. Krugelis (1946), using the histochemical method of Gomori, also demonstrated a strong rise in alkaline phosphatase activity in the nuclei at the onset of visible differentiation. The Gomori technique has, however, been subjected to severe criticism (Gomori, 1951). The intensified Feulgen reaction and the increased nucleolar activity (L. Monné, unpublished), however, support the conclusion of increased nuclear activity at the onset of visible differentiation.

These observations may be summarized in the following hypothesis. During cleavage stages, when the activity of the nuclei is mainly centred upon self-reorganization (cf. Zeuthen, 1951), mitochondrial precursors develop in the cytoplasm. When the cell division ceases the nuclear activity controls the transformation of these precursors into enzyme-rich basophilic mitochondria. The precursor development is favoured by the animal metabolism. By checking the animal metabolism, lithium thus checks the mitochondrial development. The primary point of attack is, however, not settled, neither with respect to its nature nor to its intracellular localization. An attack via the nuclei is not excluded (Gustafson & Lenicque, 1952).

The view that mitochondria may arise *de novo* and not merely reduplicate by division is supported by experiments of Harvey (1946). These show that mitochondria-free fragments obtained by centrifugation may develop into plutei. Mitochondria did not reappear in early cleavage stages, but were demonstrated in the pluteus stage. Studies by Chantrenne (1947) and Jeener (1948) on the enzymatic properties of intracellular granules of various sizes indicate a gradual elaboration of mitochondria from smaller elements. Such a concept may also be favoured by electron microscopy studies on *Tubifex* development (Lehmann, 1950).

The development of virus in a cell may serve as a model for mitochondrial development (cf. Hershey, 1952; Sanders, 1952). The *T*2 virus of *Bacterium coli*, the virus of encephalomyelitis as well as that of herpes in nerve-cells, behave in a similar way. When entering a susceptible cell the virus particles break down into smaller units. These units act as organizing centres for the formation of replicas and of further biologically 'inert' constituents. The actual synthesis of virus constituents probably occurs early in the constant phase of the growth cycle. It is followed by a phase of assembly of already synthesized subordinate units into the complete infective virus of the extracellular phase. Serological studies show that the reappearance of antigenicity and the haemagglutinative capacity of virus may precede the return of infectivity. Thus the assembly phase may take place in a series of stages. The attainment of infectivity in the phage can be correlated with the appearance of a proteinaceous membrane and a tail. The analogy between virus and mitochondrial development is disturbed by the fact that the mitochondria evidently develop during two phases with a very different degree of nuclear control.

The course of the growth cycle of a virus is largely dependent on the criteria used for measurements, i.e. whether one chooses serological properties, infectivity, cytological manifestations, or turnover rates of the infected cell. Metabolic studies may thus reveal intense growth even during the 'silent phase'. This also applies to mitochondrial development. The exponential respiratory rise during the cleavage stages of the egg may thus be linked to the early development of granules. This may also be reflected by the increased metabolic rate of purines and pyrimidines and the augmented rate of $C^{14}O$ incorporation which are both synchronous with the respiratory rise (Hultin & Wessel, 1952; Hultin, 1953).

Mitochondrial counts not only provide information about the changes in total mitochondrial number but also about mitochondrial distribution. This is uniform up to the mesenchyme blastula stage. Thereafter an animal-vegetal gradient appears. This gradient becomes steeper as development proceeds. After lithium treatment the mitochondrial-rich region extends less far than in a normal larva, whereas the reverse phenomenon characterizes the larva animalized with iodosobenzoic acid. The mitochondrial curves for isolated animal and vegetal halves resemble those of the animalized and vegetalized whole eggs. Implantation of micromeres into an animal half transforms the curve into that characteristic of a normal or a vegetalized larva, depending upon the number of micromeres implanted (Hörstadius, Lenicque, & Gustafson, 1953).

According to Runnström's double-gradient concept the animal region of the egg is the seat of animal metabolic processes. Their intensity decreases in the direction of the vegetal pole. There also occurs an oppositely directed vegetal metabolic gradient. The metabolic types are antagonistic and the results of their competition determines the extent of the primary germ layers. The animal metabolism evidently favours mitochondrial development, whereas the action of the vegetal metabolism is inhibitory. In this way a primary mitochondrial gradient may be established. Iodosobenzoic acid appears to remove the vegetal inhibitory activity, thus making the gradient less steep than normally (Gustafson & Lenicque, 1952).

The simple primary mitochondrial gradient, however, becomes complicated as development proceeds. This can be studied in a lithium-vegetalized larva (Gustafson & Lenicque, 1952). Secondary and tertiary peaks gradually appear, whereas there is a regression of the primary one. This development of mitochondria in the vegetal area may indicate a gradual defeat of the vegetal inhibitory activity. The emancipation of the mitochondrial activity does not, however, proceed straightforwardly in animal-vegetal direction, but a 'mitochondrial vacuum', the presumptive proctodaeum, persists long after mitochondria have appeared in more vegetal parts, such as the stomach. Finally, mitochondria develop in the proctodaeal rudiment too. It thus appears as if the ectodermal part or any part with a rapid mitochondrial development suppresses a similar process in the adjacent areas. This suppression can be discussed in terms of competition between different areas for a diffusible substrate or in terms of excretion of toxic waste products from the active cells. After iodosobenzoic acid treatment the primary vegetal inhibition is abolished and thereby the incitement of a gradually complicating pattern.

When cells in the larva become rich in mitochondria they begin to stretch from a cylindrical to a more flattened shape. In this way the Li-treated blastula becomes fractionated into a series of vesicles the first of which is the ectoderm. In an iodosobenzoic acid treated larva where the mitochondrial distribution is more uniform the stretching of the cells will be more or less simultaneous. A stereoblastula then arises, which we call ectodermic. The dorsoventral organization of the egg, which may manifest itself before fertilization by an asymmetric shrinkage upon the addition of certain agents, evidently disturbs and complicates the mitochondrial pattern, thus favouring mitochondrial development on the oral side (Gustafson, 1952).

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Influence of Implanted Micromeres on Reduction Gradients and Mitochondrial Distribution in developing Sea-urchin Eggs

by SVEN HÖRSTADIUS[1]

From the Zoological Institute, Uppsala

CHILD (1936) has described reduction gradients in different stages of developing sea-urchin and starfish larvae. His results concerning the gradients in sea-urchin mesenchyme blastulae and early gastrulae have been confirmed with Janus green. There exists a stronger vegetal (acropetal) reduction gradient and a weaker animal one, appearing later, in the normal whole larva. Isolated vegetal halves show a similar vegetal reduction gradient, but in most cases no animal gradient. In isolated animal halves the reduction starts in the thick plate of the enlarged apical tuft and no vegetal gradient appears. Micromeres implanted laterally in the animal part of a whole egg induce there a reduction centre. Four micromeres implanted into an isolated animal half induce a new vegetal reduction gradient and restrict the animal gradient, so that the reduction gradient system now resembles that of a normal egg. A comparison of isolated animal and vegetal halves shows that the reduction seems to start at the same time and to proceed simultaneously in both kinds of halves. We can, therefore, state that the metabolism in isolated animal halves is not identical with that of the same material when forming the animal part of a normal larva. A comparison of the reduction gradients in these different cases shows a remarkable conformity with the animal and vegetal morphogenetic gradients known from the analysis by fragmentation and transplantation experiments. (These results have been published in full, Hörstadius, 1952.)

Lindahl (1936) has suggested that the animal principle in the sea-urchin egg is connected with carbohydrate metabolism, whereas the vegetal one in later stages might be characterized by a breakdown of proteins. Gustafson (1952) has contributed evidence that mitochondria contain a series of enzymes involved in protein synthesis. The animal metabolic type favours the development of mitochondria whereas the vegetal produces inhibitors for mitochondrial development. In a normal late blastula or early gastrula the mitochondria thus decrease in number from the animal towards the vegetal pole. In corresponding

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stages of eggs animalized by iodosobenzoic acid the distribution is more uniform, due to the weakening of the vegetal metabolism. In Li-treated larvae the gradient is depressed, particularly in the more vegetal regions (Gustafson & Lenicque, 1952).

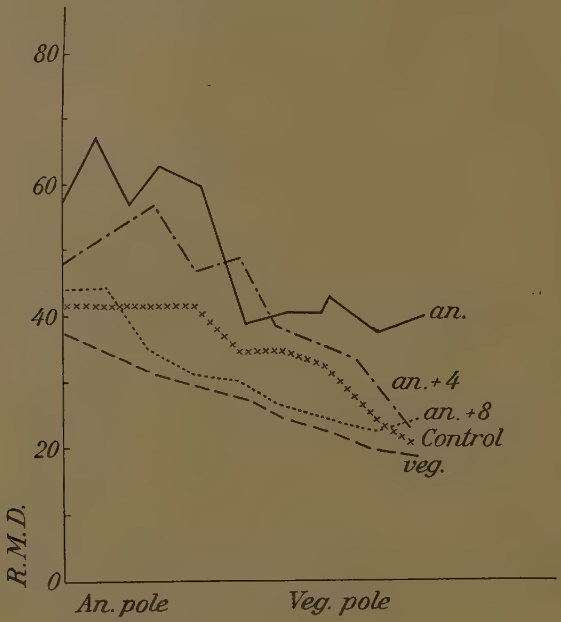

FIG. 1. Curves showing the mitochondrial distribution along the animal-vegetal axis in late blastulae–early gastrulae of sea-urchins (*Control*), in isolated animal (*an.*) and vegetal halves (*veg.*) and in animal halves with 4 (*an.*+4) or 8 implanted micromeres (*an.*+8). R.M.D., relative mitochondrial density. From Lenicque, Hörstadius, & Gustafson, *Exp. Cell Res.* (in press).

Dr. Gustafson, Mr. Lenicque, and I have made experiments to investigate whether, in analogy with the induction and inhibition of reduction gradients, differences in the distribution of mitochondria also occur when comparing whole eggs, isolated animal halves, and vegetal halves as well as animal halves with implanted micromeres.

The results are shown in Fig. 1. The mitochondrial curve of animal halves (*an.*) not only lies at a higher level but also differs in shape from that of a whole egg at a corresponding stage (*Control*). The curve of isolated vegetal halves (*veg.*) is found at a still lower level than that of control eggs and it is less complicated in form, nearly a straight line. The depressing power of the vegetal principle on the mitochondrial gradient is clearly demonstrated by implanting micromeres into isolated animal halves. The addition of four micromeres (*an.* +4) results in a curve lying between those of an animal half and of a normal egg. If eight micromeres have been implanted (*an.* + 8) the curve lies lower than that of the controls and resembles more that of vegetal halves.

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Some Experiments on Sea-urchin Eggs with Desoxynucleic Acids from Sea-urchin Sperms

by SVEN HÖRSTADIUS[1]

From the Zoological Institute, Uppsala

DR. I. JOAN LORCH, of King's College, London, and I have made some experiments on sea-urchin eggs with desoxynucleic acids (DNA) prepared from sperms of several sea-urchin species by Professor Erwin Chargaff, of Columbia University, New York.

Unfertilized eggs did not react when put into a solution of DNA in sea-water. Injection of a small amount of DNA dissolved in Callan's solution had the following consequences. If the DNA did not mix with the cytoplasm but remained as a distinct droplet, the egg could be fertilized. The droplet moved slowly towards the surface and ran out of the egg. This sometimes only occurred after several cleavages. Such eggs developed normally. If, on the other hand, the DNA mixed with the cytoplasm the egg became activated. A fertilization membrane was raised. The surface layer in dark field changed in colour from yellow to white as is the case upon fertilization. But sooner or later the cytoplasm precipitated. Lower concentrations did not cause the raising of a membrane but prevented subsequent penetration of sperm and caused precipitation of cytoplasm. The very lowest concentrations, however, did not prevent a subsequent fertilization and a normal development. The time between injection and raising of a membrane was as a rule less when higher concentrations were injected. The precipitation time varied as a rule inversely with the concentration.

The initial idea was to test if DNA from the same species was less injurious than DNA from more or less distantly related species, but the problem could not be solved because of the impossibility of measuring the exact amount of solution injected.

Animal halves of *Paracentrotus lividus* were reared in different concentrations of DNA in sea-water. DNA of the same species as well as of *Arbacia lixula* and *Echinocardium cordatum* were used. All three caused a definite animalization. No injurious effect was found with DNA acting on eggs of its own species, which is contrary to the statement made by Mazia about amphibian and starfish eggs.

Experiments were also made with synthetic nucleoside solutions. The substances were put at our disposal by Dr. Jack J. Fox and brought from the Laboratoire de morphologie animale in Brussels (director, Professor Jean Brachet).

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Animalization was achieved with glucopyranosylcytosine, xylopyranosylcytosine, and arabinopyranosylcytosine, whereas tests with galactopyranosylcytosine and galactopyranolsyluracil did not lead to any change in differentiation. It is of interest that some of these substances, analogous to breakdown products of DNA, have a similar effect to DNA.

It should, perhaps, finally be pointed out that we have in desoxynucleic acid a substance causing both activation and animalization.

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Intercellular Relations in the Development of Amphibian Pigmentation

by VICTOR C. TWITTY[1]

From the Department of Biological Sciences, Stanford University

THE role of intercellular influences in the control of differentiation finds many illustrations in the development of amphibian pigmentation. From the time the chromatophores leave the neural crest as colourless, amoeboid cells, until they come to rest as differentiated elements of the distinctive pigment pattern, their developmental behaviour is conditioned throughout by effects exerted mutually by the chromatophores themselves or imposed upon them by the cells of neighbouring tissues. For the purposes of the present discussion, I shall limit myself to examples of intercellular relations affecting (1) the migration of chromatoblasts and (2) their synthesis of pigment.

PIGMENT-CELL MIGRATION

Various suggestions have been made concerning the nature of the factors responsible for the dispersion of pro-pigment cells from the mid-dorsal line to their resting-places throughout the skin. One is that the cells are actually drawn ventrally in response to a true chemotactic attraction exerted by the skin beneath which they spread. It has also been suggested that the cells follow ultramicroscopic physical pathways present in the skin. However, my own experiments tend to discount the importance of these factors, and have led me to propose that the cells scatter from the neural crest in response to what we may term their own population pressure. Specifically, it seems that these cells release chemical substances which have an excitatory effect on their neighbours and cause the cells to scatter until they are beyond the range of this mutual influence or repulsion. Until quite recently almost all of the evidence in support of this view has been derived from experiments with relatively large populations of cells in tissue culture (Twitty & Niu, 1948; Twitty, 1949). For example, chromatoblasts were cultured under conditions of extreme spatial confinement in order to favour the accumulation of the postulated excitatory products in higher and accordingly more effective concentrations. At one side of the explant of neural crest, in a drop culture, a low-ceilinged chamber was constructed by supporting a small fragment of cover-slip a short distance above the primary cover-slip. Cells migrating from the explant into this confined space beneath the fragment moved more rapidly

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and became more widely spaced from one another than those spreading elsewhere on the cover-slip, where any diffusible products released by the cells would escape more freely from their immediate vicinity. This experiment, together with various modifications of it, leaves no doubt that chromatoblast migration is motivated in considerable degree by intercellular effects mediated through diffusible products. Moreover, similar results with chick fibroblasts and cells of subcutaneous mouse tumour show that the movements of other types of 'mesenchymatous' cells respond to the same influences that excite chromatophore migration.

If cell migration is dependent upon mutual stimulation between neighbouring cells, it should follow that a single cell, in the absence of any neighbours, would be unable to execute migratory excursions. Accordingly during the past two years this has been tested by experiments of the type illustrated schematically in Fig. 1. One or more cells are drawn from a young culture of neural crest into the capillary tip of a mouth pipette, and the tip broken off and secured in position by embedding one end in a mass of vaseline on the floor of a Petri dish. In this way several tubes may be accommodated in a single dish. The tubes themselves contain coelomic fluid, obtained from the body cavity of adult salamanders, since a natural medium of this type proves to be much more favourable for the survival and differentiation of the isolated cells than is physiological saline. However, once the tubes are in position, the dish is filled with physiological salt solution and then sealed with a glass cover.

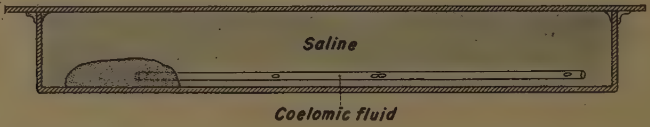

FIG. 1. Diagram illustrating conditions under which pro-pigment cells are isolated in capillary tubes of very small diameter. The tubes are very much longer relative to cell-size than indicated by the diagram.

The essence of the results can be summarized by reference to the diagram. Single cells situated at any place along the length of the tube except near the open end remain essentially stationary. They undergo active changes in shape and differentiate normally, but any migratory excursions are of almost negligible extent. When isolated in pairs, the cells characteristically move away from one another, often for considerable distances, and the strongly directional character of their movements indicates that they are moving in response to concentration gradients. The same behaviour is shown by groups of three or more cells, and in general there is a close proportionality between the number of cells in the group and the total distance over which they become distributed.

The behaviour near the open end of the tube is somewhat different, but is nevertheless still in keeping with the idea that the movements of the cells are susceptible to concentration gradients. Single cells situated near the open end tend to move deeper within the tube, probably in response to concentration gradients resulting from the exchange of diffusible substances between the coelomic fluid within the tube and the physiological salt solution without. Small groups of two or three cells are also often sensitive to this concentration gradient, but as they move deeper within the tube they also tend to become more widely spaced in response to their own mutual repulsion. And when larger aggregates are present, this latter force will in fact drive some of the cells closer to the open end.

DIFFERENTIATION OF THE PRO-PIGMENT CELLS INTO CHROMATOPHORES

Some of the most striking evidences of intercellular effects on chromatophore development come from experiments on their chemical differentiation, i.e. the synthesis of the specific pigment characteristic of melanophores, xanthophores, or guanophores. It is clear that the chromatoblasts are not autonomous pigment-building units, but are instead heavily dependent upon extrinsic influences, including influences exerted mutually by the pro-pigment cells themselves. Most readers are familiar with the experiment of exchanging epidermis between young embryos of the black axolotl and of the albino axolotl. It is clear from the results of such experiments that the albino axolotl is not deficient in melanophores because of any lack of potential melanoblasts, but because the environmental tissues of this mutant form fail to provide an adequate environment for the differentiation of pro-pigment cells. For example, when a portion of the flank ectoderm is replaced by epidermis from a black axolotl embryo, the pro-pigment cells subsequently migrating from the host neural crest and coming to lie beneath the transplant develop readily into fully differentiated melanophores, forming a heavy patch of black pigment that contrasts conspicuously with the sparsely pigmented skin of the rest of the host (DuShane, 1935). When both ectoderm and underlying mesoderm are replaced, the increase in pigmentation is even more striking (DuShane, 1939).

Although some disagreement has been expressed (Dalton, 1950), there is considerable reason to believe that the environmental tissues effect control of melanoblast differentiation, at least in part, by contributing—or failing to contribute—pigment precursors or enzymes known to be essential to the synthesis of melanin. On the assumption that this is the case, I should like to present evidence that the pro-pigment cells compete with one another in acquiring these essential substances from the ectoderm and mesoderm. There is a variety of evidence pointing to the role of competition between melanoblasts as a factor affecting the size of the melanophore complement in salamander larvae, but I shall choose for illustration one particular group of experiments.

The first of these involves the creation of embryos with chimaeric neural crest, by replacing one neural fold of a salamander embryo with a fold from a donor of different species (Twitty, 1945). Since the two neural folds are the anlagen of the neural crest, closure and fusion of the grafted and host folds results in the formation of neural crest of composite genetic constitution, one-half from the resident host fold and one-half from the grafted alien fold.

If the donor and host species are of closely similar developmental rate, as, for example, when *Triturus torosus* and *Triturus rivularis* are employed for cross transplantation, the results of the experiment present no unusual or unexpected features. The composite neural crest, in which the cells of the two species apparently become quite thoroughly intermixed, sends chromatoblasts of both species to both flanks, where subsequently *torosus* and *rivularis* chromatophores differentiate in approximately equal numbers.

The outcome is strikingly different, however, if donor and host species differ substantially in inherent developmental rate, as when a fold of *Triturus torosus* is replaced by another from an embryo of one of the rapidly developing species of *Amblystoma*, such as *mexicanum*, *tigrinum*, or *californiense*. The results vary slightly with the donor species employed (Lehman, 1950), but in all cases the grafted chromatoblasts assume dominance over those of the host. Both flanks become populated almost completely with *Amblystoma* pigment cells, to the virtual exclusion of chromatophores of host type. This phenomenon has been tentatively attributed to an advantage enjoyed by the *Amblystoma* chromatoblasts in competing for available pigment precursors or enzymes with the less rapidly developing pro-pigment cells of the host. Initiating their migration and differentiation earlier than the pro-pigment cells of host origin, they are in position to forestall development of the latter by pre-empting essential pigment-promoting substances supplied by the environmental tissues.

This thesis has recently been subjected to further tests by a student in my laboratory, Mr. Robbins King. Before recounting his experiments, it should be explained that the pigment-promoting capacity of the ectoderm and mesoderm of *T. Torosus*, although stronger than that of the same tissues of the albino axolotl, is nevertheless limited in comparison with other pigmented forms, such as the species of *Amblystoma* just mentioned. Thus when a piece of flank epidermis is removed from a *torosus* embryo and replaced by epidermis from a *tigrinum* or black axolotl embryo, the number of melanophores subsequently differentiating beneath the grafted epidermis far exceeds the normal *torosus* complement (Twitty, 1945; DeLanney, 1941).

In view of this relative weakness of the pigment-promoting capacity of the *torosus* tissues, it follows that in the *torosus* embryos with chimaeric neural crest the competitive situation is especially keen, i.e. the *torosus* and *Amblystoma* pro-pigment cells are competing for a very limited supply of substances essential to melanin synthesis. This raises the following question. Would the *torosus* pro-pigment cells in these chimaeric embryos be at a less extreme competitive disadvantage if the pigment-forming substances afforded by the environmental tissues could be made available in a higher, less restrictive concentration? One obvious means of testing this is to create a *torosus* embryo with chimaeric crest, as before, and then to enhance the pigment-promoting capacity of the environment by replacing portions of the host tissues (epidermis and / or mesoderm) with corresponding pieces from *Amblystoma.* Under these conditions of greater 'abundance' it is conceivable that the *torosus* pro-pigment cells might have an opportunity to obtain a more significant share of the substances requisite for pigment synthesis.

When epidermis alone was grafted to the flanks of chimaeric hosts, the result was merely to increase the number of *Amblystoma* pigment cells, in the graft region, without promoting the differentiation of additional host chromatophores; apparently the environment for pigment development, although improved by this experiment, still fails to saturate the demands of the more avid grafted cells.

However, when both ectoderm and the underlying somite mesoderm of the chimaeric host are replaced by the corresponding *Amblystoma* tissues, differentiated host melanophores now emerge in substantial numbers. *Amblystoma* chromatophores are, of course, also increased in number and continue to constitute a great majority of the total population, but apparently the environment is now sufficiently rich to relieve the competitive pressure and afford the *torosus* cells at least a modest share of pigment-building materials.

Of particular interest and promise are the results of preliminary experiments in which chemical methods are brought to bear on the problem of competitive interplay between developing chromatophores. As is now well established, certain anti-thyroid drugs have the property of strongly inhibiting melanin synthesis. In the course of a comparative study King found evidence of significant species differences in sensitivity to the inhibitory effects of these drugs. For example, melanin formation was completely suppressed in both *A. tigrinum* and *T. torosus* embryos when grown in 0·005 per cent. solutions of phenylthiourea, but when the concentration was reduced to 0·002 per cent. the two species responded differentially. The distinction was not always marked, because of slight individual variations in sensitivity within each species, but the average degree of pigmentary inhibition was definitely greater in *A. tigrinum.* Frequently larvae of this species were completely lacking in melanin, and at most there were a relatively few palely pigmented melanophores; while in *torosus* some melanophores were always evident and usually they were present as a substantial, almost completely pigmented, complement.

The existence of this difference in sensitivity to phenylthiourea suggested performance of the following experiment. Single folds of *torosus* neurulae were replaced with folds from *tigrinum,* as before, but now the young hosts were placed in solutions of phenylthiourea of the critical concentration just indicated. Remembering that in the absence of phenylthiourea such hosts become populated almost exclusively with *tigrinum* melanophores, the question arises whether with virtually complete suppression of pigment synthesis by these cells the *torosus* melanoblasts may be partly freed from their competitive handicap and accordingly emerge in larger numbers.

The results show that this possibility is actually realized. In about 30 per cent. of the cases *torosus* melanophores appeared in numbers exceeding those ever observed in hosts reared in ordinary media. Under the latter conditions, all except 2 or 3, or at most 8 to 10, *torosus* melanophores are suppressed, whereas with differential inhibition of the *tigrinum* melanophores by phenylthiourea the average number is definitely greater and may be as high as 30 or 40. Considerable variation in the number of *torosus* melanophores is probably to be expected in these experiments, since, as mentioned above, the degree of sensitivity to phenylthiourea varies within each species. The most striking cases are probably the result of combining crest from individuals of *torosus* and *tigrinum* presenting maximal species differences in sensitivity, while in cases in which an increase in the number of *torosus* melanophores is small or lacking, one may assume that the donors and hosts were specimens of comparatively similar sensitivity.

Although these experiments require further elaboration, their implication seems clear, namely, that the distribution of pigment precursors between chromatoblasts is indeed effected on a competitive basis, and that selective interference with their utilization by one part of the population automatically increases the supply available to the remainder.

SUMMARY

The role of intercellular influences in embryonic differentiation is illustrated by two examples from studies on the development of amphibian pigmentation. (1) The dispersion of pro-pigment cells from the neural crest is motivated by mutual stimulation, through excitatory effects exerted by diffusible substances produced by the chromatoblasts themselves. (2) Evidence is presented that pigment synthesis within the chromatoblasts is dependent upon chromogens or enzymes contributed by the environmental tissues (epidermis and mesoderm), and that the pro-pigment cells compete with one another in obtaining these essential materials.

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Determination of Cell Function in an Insect

by V. B. WIGGLESWORTH[1]

From the Department of Zoology, University of Cambridge

I PROPOSE to consider two kinds of determination and differentiation which have been studied in the hemipteron *Rhodnius prolixus*. (i) The determination of the cell or group of cells, with their subsequent differentiation to produce a given part of the body. (ii) The determination or control of the characters of that part—whether these are to be juvenile (larval) or adult (imaginal). Discussion of this second type of determination will require consideration of the role of hormones in controlling differentiation in insects.

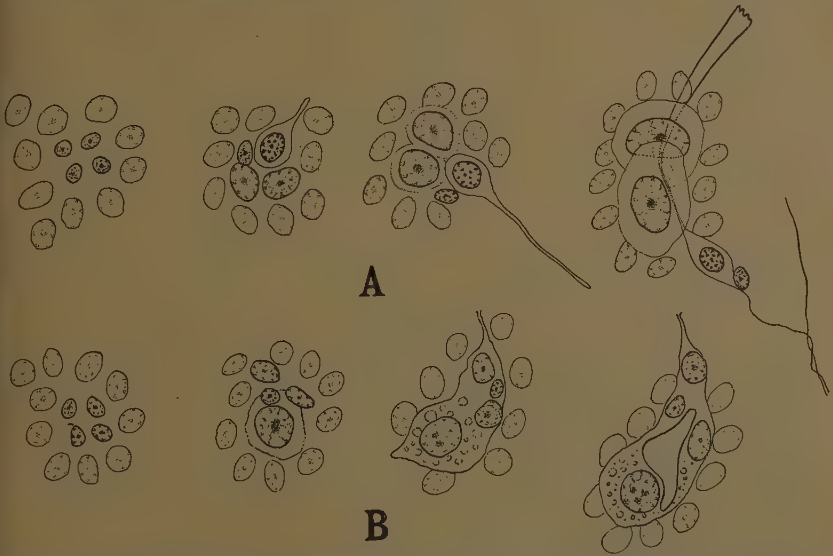

FIG. 1. A, four stages in the development of a sensory hair showing four small cells becoming differentiated into the trichogen, tormogen, sense-cell with inwardly growing axon, and neurilemma cell. B, four stages in the development of a dermal gland by the differentiation of four small cells.

DETERMINATION AND DIFFERENTIATION OF NEW STRUCTURES

The integument of the abdomen in the *Rhodnius* larva consists of a single layer of epidermal cells and the overlying cuticle. At regular intervals the cuticle

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is modified to form little plaques each of which bears an innervated bristle (Wigglesworth, 1933). The cuticle is pierced at intervals by the ducts of dermal glands: these form a cluster of 4 or 5 around each plaque, with occasional single glands in the clear space between (Wigglesworth, 1947) (Fig. 3, A).

At each moult bristles and plaques are re-formed at the existing sites and new ones are developed in the spaces between (Wigglesworth, 1940*a*). The new bristle first appears as a group of four apparently identical cells with small nuclei, doubtless the product of rapid division of a single cell which has been determined as a bristle-forming centre (Fig. 1, A). These four cells become the trichogen, the tormogen, the sense-cell which sends an axon inwards to join with neighbouring axons, and the neurilemma cell (Wigglesworth, 1953).

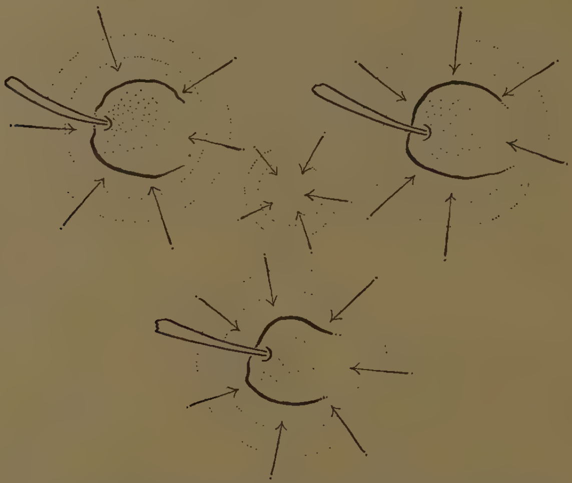

FIG. 2. Diagram to illustrate the hypothesis of determination of a new sensory hair in the interval between existing hairs.

At their earliest appearance the dermal glands likewise arise as four apparently identical cells with small nuclei which later become differentiated into a large glandular cell, two cells with smaller nuclei, and a cell with a very small nucleus resembling the neurilemma cell (Wigglesworth, 1933, 1953) (Fig. 1, B).

The determination of these new structures is controlled by their distance from the existing structures. New bristles appear where the existing bristles are most widely separated. If the epidermis is destroyed by burning over a wide area, new bristles are regenerated at the normal intervals. If the existing bristles are abnormally separated by stretching the integument experimentally (for example, by blocking the anus after a large meal of blood), an unusually large number of new bristles appear between them (Wigglesworth, 1940*a*).

It would seem that existing bristles inhibit the emergence of new bristle-forming centres for a certain distance around them. The epidermal cells are intimately connected together by means of anastomosing processes below the basement membrane so that they form in effect a syncytium (Wigglesworth, 1937, 1953). As a working hypothesis (Fig. 2) it is considered that some substance present in the epidermis is necessary for the determination of a bristle-forming centre. Each centre drains this substance from the surrounding cells. Hence it is at a point as far removed as possible from existing centres that the new centre will arise; and this in its turn will inhibit the appearance of further centres in its vicinity by drawing off the hypothetical substance (Wigglesworth, 1940*a*, 1948*b*).

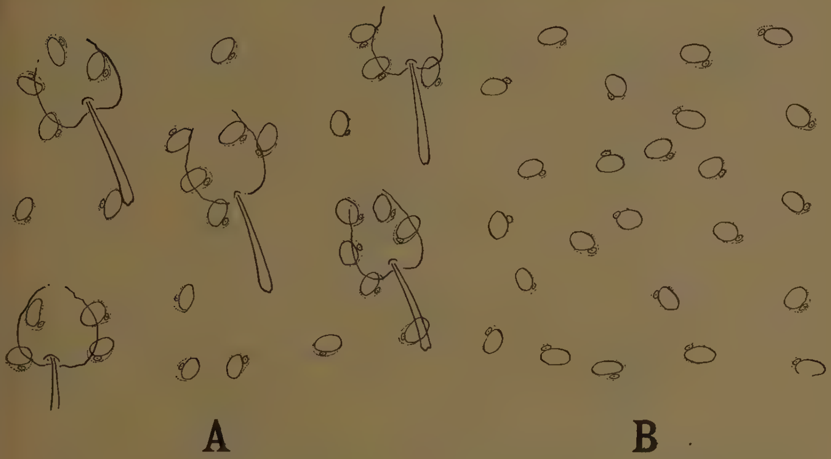

FIG. 3. A, normal integument showing distribution of sensory hairs and dermal glands. B, integument at the first moult after the repair of a burn; only dermal glands have been regenerated.

In the first moult after burning no new bristles are formed in the regenerated area; they appear at the second moult. But examination of the regenerated epidermis after a burn shows that new dermal glands *have* been differentiated and at regular intervals, much closer together than the bristles (Wigglesworth, 1953) (Fig. 3, B).

There is no obvious reason why new bristles should not be regenerated at the first moult after the repair of an extensive burn—for during the early stages in their differentiation the bristles are indistinguishable from the developing dermal glands (Fig. 1), and we have seen that dermal glands with normal intervals between them are developed even at this stage. One might put forward the hypothesis that the same substance is necessary to evoke the differentiation of sensory bristles and dermal glands but that it is required in higher concentration for the production of sensory bristles. One might then suppose that the concentration of this substance in the epidermis of a recently healed burn is insufficient for the formation of bristles but adequate for the differentiation of glands. An hypothesis of this kind would also explain the fact that glands develop closer together than bristles.

New bristles arising over a healed burn are normally orientated; that is, they are directed posteriorly; whereas if a piece of the integument is excised, rotated through 90° or 180° and reimplanted, the bristles show a corresponding change in orientation (Wigglesworth, 1940*b*). One must therefore conclude that some sort of orientation already exists within the cytoplasm of the undifferentiated epidermal cells, with the result that when they divide to produce the tormogen and trichogen cells these are so placed in respect to one another that the bristle grows out in the predetermined direction.

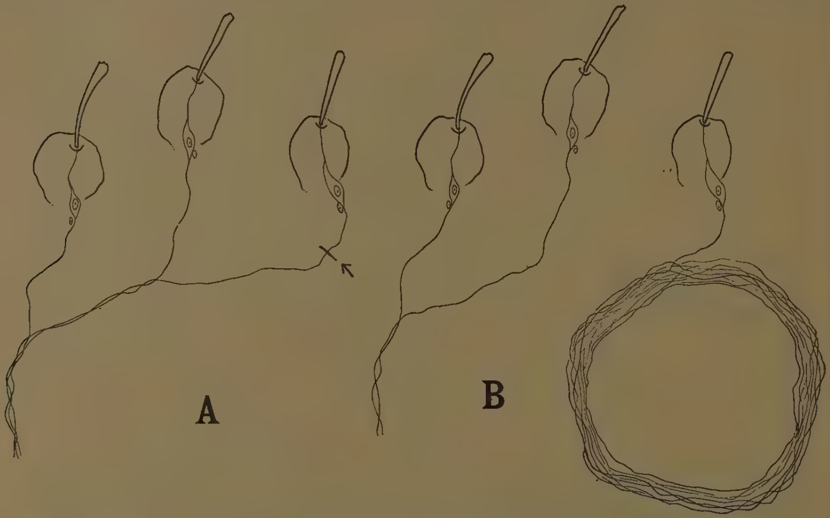

FIG. 4. A, three sensory hairs with their axons forming a small nerve. The arrow shows point of interruption of one axon by a burn. B, the same after repair. The outgrowing axon has formed an annular nerve with no connexion with the central nervous system.

These observations point to the existence of some kind of 'cytoskeleton' within the undetermined cell, which defines the antero-posterior axis and controls the mutual relations of the daughter cells (themselves perhaps still forming a syncytium) and in this way controls the orientation of the resultant structures—just as a unicellular protozoan may have a highly differentiated structure.

The new sensory neurones arise by differentiation from the ordinary ectodermal cells throughout post-embryonic life. Their inwardly growing axons illustrate another common phenomenon in the relations between differentiated cells: the mutual affinity of the same cell types. The new axon may grow in any direction, between the epidermal cells and the basement membrane, until it makes contact with an existing nerve or axon. It then proceeds to accompany this nerve, which it follows through the basement membrane and to the central nervous system. If an existing sensory nerve is cut or interrupted by a burn, the axons grow out during the healing process in the same manner and accompany any nerve they chance to meet. Sometimes, however, they will grow round and make contact with themselves and will then proceed to grow round and round in circles until a stout annular nerve is produced which has no connexion with the central nervous system (Wigglesworth, 1953) (Fig. 4).

DETERMINATION AND DIFFERENTIATION OF JUVENILE AND ADULT CHARACTERS

Some insects, notably Diptera, have the imaginal cells set aside at an early stage of development. But in *Rhodnius* the same epidermal cells are responsible for forming both larval and adult structures. When the cells are determined to form some particular region of the body in the larva they are committed to form the corresponding region of the adult.

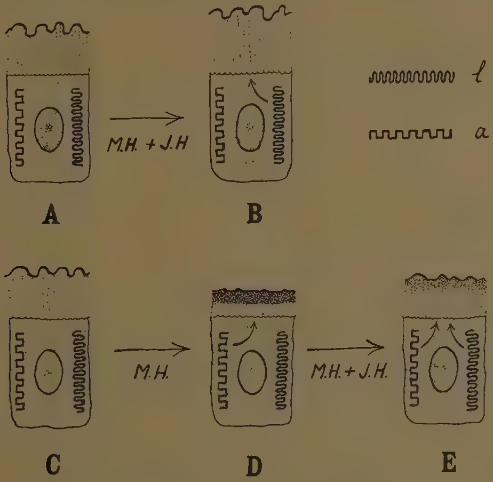

FIG. 5. Diagram to illustrate the control of metamorphosis by hormones. *a*, intracellular system producing adult characters; *l*, intracellular system producing larval characters. Epidermal cell with larval cuticle (A) in presence of moulting hormone (M.H.) and juvenile hormone (J.H.) lays down larval cuticle (B). Similar epidermal cell (C) in presence of moulting hormone alone (M.H.) lays down adult cuticle (D). In presence of moulting hormone and juvenile hormone it now lays down cuticle intermediate between adult and larva (E).

The morphological characters of the cuticle may be totally different in larval and adult stages; which characters appear is determined by the hormones in the circulating blood. Growth and moulting are initiated by hormones from the brain and thoracic gland. In the presence of these hormones (the 'moulting hormone') alone the intracellular system which lays down adult structures takes precedence over that laying down larval structures (Fig. 5, D). But if the juvenile hormone secreted by the corpus allatum of the young larval stages is present in addition, the system which lays down larval structures is preferentially activated and metamorphosis is therefore suppressed (Fig. 5, B) (Wigglesworth, 1934, 1936, 1940*b*).

The change in function of one and the same cell is seen most strikingly in the form of the bristles. These are produced by outgrowths from a single trichogen cell which persists from the larva to the adult. By appropriate manipulation of the growth-controlling hormones it is possible to produce *Rhodnius* larvae showing all intermediate degrees of metamorphosis between larva and adult. The corresponding changes are manifested in the individual bristles (Wigglesworth, 1934) (Fig. 6).

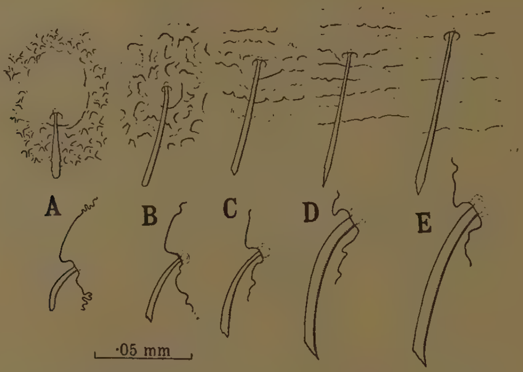

FIG. 6. Bristles from dorsal surface of abdomen (upper row) and from margin of abdomen (lower row). A, normal fourth-stage larva; B, C, and D, insects showing varying degrees of metamorphosis following removal of corpus allatum from third-stage larva; E, normal adult.

Even in the adult *Rhodnius* the system which produces larval structures is still latent within the cells. If the adult is caused to moult again by exposure to the 'moulting hormone', and if at the same time it is provided with a source of juvenile hormone, it shows a partial reversion to larval characters (Fig. 5, E). In the light of these results it is not wholly satisfactory to refer to the juvenile hormone as the 'inhibitory hormone', restraining the differentiation of imaginal characters by sustaining the differentiation of larval characters (Wigglesworth, 1934) or as the *status quo* hormone (Williams, 1952) maintaining the larval characters by restraining imaginal differentiation. This hormone must be pictured as having a more *positive* action on the latent morphological system in the cells, and as actively favouring differentiation by the larval system even after metamorphosis has taken place (Fig. 5, E). For that reason the term 'juvenile hormone' (Wigglesworth, 1940*b*) is perhaps to be preferred.

It may be pointed out that the 'moulting hormone' (which consists of a tropic factor from the brain activating an endocrine thoracic gland which then secretes the hormone acting on the tissues) (Wigglesworth, 1952*a*; Williams, 1948, 1952) has been alternatively named a 'growth and differentiation factor' (Scharrer, 1946). But the results outlined above make it clear that differentiation may take place in the larval direction or in the imaginal direction. The 'moulting hormone' merely initiates and maintains the process of growth which terminates in moulting. The capacity for dimorphic differentiation is a property of the growing body cells, the *direction* of differentiation being controlled by the concentration (Wigglesworth, 1934) and by the timing (Wigglesworth, 1952*b*) of the secretion

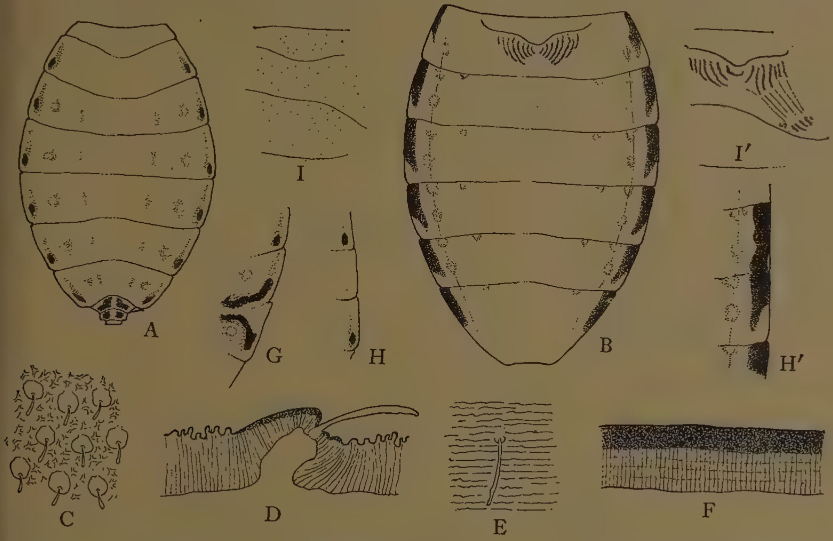

FIG. 7. A, dorsal abdominal pattern of *Rhodnius* larva; B, the same, *Rhodnius* adult; C, detail of surface of cuticle in larva, showing plaques and bristles; D, section through cuticle of larva; E, detail of surface of cuticle in adult showing transverse folding of epicuticle and few bristles; F, section through cuticle of adult; G, margin of abdomen of fifth-stage larva after repair of a burn involving two marginal black spots (note extension inwards of the black spots concerned); H, elimination of one marginal black spot in fifth-stage larva as result of a burn in this area during third stage; H′, the corresponding area when this insect became adult (the marginal black spot of that segment in the adult has extended over the burned area); I, first three segments in fifth-stage larva following an extensive burn on the right side in the third stage (apart from some distortion of the second segment there is no visible effect); I′, corresponding area when this insect became adult (there is a striking displacement of the ridged cuticle on the second segment).

of the juvenile hormone. There is, therefore, no justification for ascribing to the 'moulting hormone' the property of *inducing differentiation* which is what the term 'differentiation factor' implies.

The structural pattern of the abdomen is quite different in larva and adult. An area on the first and second abdominal segments, indistinguishable in the larva from the rest of the surface of the abdomen, forms in the adult a characteristic zone with semicircular ridges. Pale areas at the sides of the abdomen in the larva become black spots in the adult; while the black spots of the larva are replaced by pale areas in the adult (Wigglesworth, 1940*a*) (Fig. 7, A, B).

That the imaginal pattern already exists in invisible form in the larva is proved by eliminating areas of the epidermis by burning. Regeneration takes place by the multiplication and spreading of cells from the adjacent areas. When the insect finally undergoes metamorphosis it is found that the power of the cells to lay down particular elements in the adult pattern has been transmitted to the daughter cells and that during the process of healing the imaginal pattern has been displaced (Wigglesworth, 1940*a*).

These results are summarized in Fig. 7. The ridged cuticle in the second abdominal segment may be strikingly displaced in the adult following a burn in an early larval stage (Fig. 7, I′), although no sign of this displacement is visible in the larva (Fig. 7, I). Likewise a burn eliminating a black spot in the larval tergites (Fig. 7, H) may be repaired by cells destined to produce a black area in the adult so that two black spots in the resulting adult are found to have fused (Fig. 7, H′). Whereas a burn in the pale area between two black spots in the larva may result in the fusion of the spots in the later larval stages, but at the same time it has eliminated the area destined to form a black spot in the adult so that this spot is absent.

It is a matter for discussion whether the simultaneous inheritance of the dual potentialities for larval and adult differentiation within these societies of cells is by way of the nucleus or cytoplasm or both.

SUMMARY

This paper reviews briefly a wide range of published observations on growth and differentiation in *Rhodnius*. Subjects mentioned include:

i. The differentiation of sensory hairs and dermal glands.
ii. The effect of mutual separation in controlling the formation of new hairs and glands.
iii. The differentiation of primary sense-cells and the behaviour of the sensory axon during growth and regeneration.
iv. The existence of larval and imaginal potencies in single epidermal cells and the role of hormones in controlling their differentiation.
v. The reversal of metamorphosis in the adult insect.
vi. The persistence of the latent potencies for particular elements in the imaginal pattern during wound healing in the larval stages.

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Histolysis, Histogenesis, and Differentiation during Insect Metamorphosis in Relation to Metabolic Changes

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THE most obvious change in metabolism during the more advanced type of insect metamorphosis is the change in the integral metabolic activity. After pupation there is first a marked decrease and later an increase of, for instance, the oxygen consumption. The respiratory metabolic curve is U-shaped. The cause of this change is a corresponding variation in the activity of oxidative enzyme systems. If one compares the variation of the spontaneous Mb-reduction and of the oxygen consumption in the fly *Calliphora*, one finds that the two curves have almost the same course (Agrell, 1947*b*). This shows an important co-operation of the dehydrogenase systems. The cytochrome system also shows a similar U-shaped variation during the period of metamorphosis, according to Wolsky (1938), Williams (1948) and Sacktor (1950). One limiting factor is the protein part of the enzymes, which is first broken down and later rebuilt (Agrell, 1946). Another limiting factor is the prosthetic heme group in the cytochromes (Williams, 1951). According to Sandborn & Williams (1950) there is also a change over from a cytochrome X, possibly identical with Keilin's cytochrome E, to cytochrome C at pupation.

The course of the metabolic curve coincides with the morphological events. This was first presupposed by Krogh (1914), but was later denied (Dobzhansky & Poulsŏn, 1935). By determination of the activity of the dehydrogenases in different parts of the pupa, of head, thorax, and abdomen separately, I was able to show that the rising part of the metabolic curve concerns, almost solely, the thorax during the time of muscle formation (Agrell, 1949*c*). Thus during the period of metamorphosis the increasing metabolism in fact represents histogenesis and the decreasing metabolism denotes histolysis. Histogenesis, however, is almost completed some time before hatching and in spite of this fact the metabolic activity continues to rise. The 'missing part' of the increasing respiratory curve can be found in the relation between non-soluble and soluble protein. During the later part of the pupal period there is obviously an increase in the amount of non-soluble protein representing an incorporation of soluble protein

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in the cellular structure, for example, a fixation of enzymes in mitochondria and thus an increased respiratory activity. From these results it seems clear that the period of metamorphosis can be regarded as consisting of three physiologically different parts: first a period of predominating histolysis, characterized by a decomposition of even the respiratory enzymes; later a period of predominating histogenesis; and lastly a period of differentiation. During these periods a resynthesis and structural arrangement of the respiratory enzymes occurs. Of other enzymes, the apyrase also shows a U-shaped curve of change, but it reappears first during the differentiation period. Concerning the nucleic acids there is an increase in amount of DNA, which tolerably closely follows the course of histogenesis (Agrell, 1952). There is also an obvious inverse variation in amount of DNA and PNA. This inverse change strongly suggests a mutual interdependence of the two nucleic acids as regards their synthesis in the pupa.

In spite of the fact that tissues, cells, and enzymes are broken down and rebuilt during metamorphosis, there is almost no change in the activity of the cell proteolytic enzymes (Agrell, 1951*b*). There is, however, a remarkable U-shaped change in tissue-pH, a decrease during histolysis, and an increase during histogenesis and differentiation. Integral pH varies not less than one pH-unit during this time (Agrell, 1948). The proteolytic enzyme found is of the peptic type (Agrell, 1951*b*), and thus it is activated with increasing histolysis. The initiation of histolysis may depend primarily upon a changed hormonal balance presumably causing a disturbed respiratory metabolism. This starts the causal sequence: accumulation of acid metabolites, activation of cell proteolytic enzymes, proteolysis of even the respiratory enzymes, further changed respiratory metabolism, and so on. The result is histolysis of the larval body. Eventually the imaginal tissues develop. The interaction between tissue-pH and destruction of respiratory enzymes can also be seen on a smaller scale during experimental anaerobiosis.

There exist varying types of metabolism during the above-mentioned physiologically conditioned periods of metamorphosis. Different pathways are utilized for energy gain. For this investigation I have used the Mb-technique and specific enzyme inhibitors: fluoride for inhibition of the glycolytic system and malonate for the tricarboxylic acid cycle. Following the inhibition of the Mb-reducing capacity of homogenized pupae I found a reciprocal variation in inhibition by malonate and fluoride respectively. A high fluoride inhibition corresponds to a low malonate inhibition and vice versa. Thus, with increasing histolysis the activity of the glycolytic system decreases and that of the tricarboxylic acid cycle increases. During histogenesis the glycolytic system eventually predominates, and finally during the period of differentiation the processes are once more reversed. Other experimental results also speak in favour of this alternative variation in activity of the two systems. If to the pupal homogenate are added different hydrogen donors, especially those concerned with the tricarboxylic acid cycle, a change in respiratory activity is obtained, which follows the variation of this cycle indicated by the malonate inhibition (Agrell, 1947*b*). In intact pupae the lactic acid content and the activity of the lactic acid dehydrogenase varies according to the above-described alternation of the two systems.

The changes of the above-mentioned enzyme systems affect the energy level in the pupa. Both the amount of the fraction ATP + ADP as of AMP varies along a U-shaped curve. During metamorphosis first a decomposition and later a rebuilding of these phosphates obviously occur. The decomposition of AMP goes farther. As mentioned, there is a U-shaped variation of tissue-pH. By paper chromatography I have found only one quantitatively prominent free acid during pupal life. Its amount varies according to the pH-curve. This acid has its absorption maximum at 2,500 A and behaves like inosinic acid; it could therefore be this decomposed, deaminated product of the adenosine phosphates. If this is so, it seems as if it is principally the decomposition of the adenosine phosphates that activates the cell proteolytic enzymes and in this way governs the events of histolysis and histogenesis. The amount of easily hydrolysable, energy-rich phosphate, 'ATP', varies differently. It increases during histolysis, decreases during histogenesis, and increases again during differentiation. This seems to indicate a decreased rate in metabolic turnover of these phosphates during the histolytic period, a relative absence of synthetic processes. It is metabolized at a higher rate during histogenesis presumably by intense synthesis, but its level increases again during the early part of differentiation, the first part of which should represent a period of relative synthetic rest. These results agree in a striking manner with the sensitivity of the pupae to experimental anaerobiosis. Concerning arginine phosphate I have observed that its amount varies similarly to 'ATP' during the metamorphosis period.

Pupae of some insect species show a spontaneous arrest in development; they enter into a so-called diapause. The diapause seems always to occur during a period of lowered metabolism and thus when the utilization of energy is low. Diapause is primarily caused by endogenous factors and is genetically fixed. It is hormonally governed, and the ultimate reason is an arrest in production of the hormonal principle from the brain. Chilling of the animal or only of the brain breaks the diapause (Williams, 1947). How the hormones control the metabolic processes we do not know. However, other biochemical and physiological data have been obtained and I will use them as a contribution to the problem of how a blocked development and differentiation may recommence. Williams (1948) lays stress upon the importance of cytochrome C as it seems to be completely absent during diapause and resynthesized during resumed development. Diapause could be caused by a temporary inability to form cytochrome C. The metabolism for maintenance could be kept going by the functioning of flavoproteins as terminal oxidases (Williams, 1951). It may be so, but no doubt other factors co-operate. The biochemical pattern is a complicated one. As I have found, another characteristic feature is the remarkably low respiratory quotient of butterfly pupae in diapause. The RQ assumes successively increased but steady levels depending upon the developmental stage (Agrell, 1947*a*). The main difference is a different thermal coefficient for RQ. It seems as if two antagonistic processes go on: one with a high RQ prevailing at and promoted by low temperature, a process of complete combustion; the other a process of conversion of an oxygen-poor compound, probably fat, into an oxygen-rich compound, probably carbohydrate. The development is activated by low temperature and thus by the process with high RQ, which should also give a higher energy gain and give the opportunity for synthetic processes, for instance, formation of the hormonal principle, which activates further development. Thus the hormone may be a biocatalyst kept in existence by the processes which it catalyses itself (Agrell, 1950). Further, there are facts which indicate that diapause has something to do with a vitamin deficiency. Diapause may be induced in the larval stage by environmental factors, which all represent a restriction of the intake of nourishment, for example, cold, decrease of daylight, kind of food, &c. The low RQ denotes a disturbed decarboxylation. Vitamins, for example, thiamine and pantothenic acid, are building-stones for enzymes working in the decarboxylating part of the tricarboxylic acid cycle, co-carboxylase, and co-enzyme A. In fact, an injection especially of thiamin causes an immediate rise in RQ (Agrell, 1951*a*). Thus it seems as if the cause of diapause is connected with the availability of these vitamins. If the decarboxylating part of the tricarboxylic acid cycle is inefficient, there is a pathway for energy gain by transforming fat into carbohydrate along the Thunberg–Wieland cycle.

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Cellular Interactions in Cell Differentiation

by RODOLFO AMPRINO[1]

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IT is of some interest, in connexion with the problem of cellular interactions, to view the *affinities* between different tissues in various stages of development.

The problem has been extensively investigated by Holtfreter in the early stages of the amphibian egg. Professor P. Weiss has briefly reported on the stimulating observations made by Chiakulas (1952) on grafts of epithelia from various sources to the skin of larval amphibians.

I have performed experiments which bear some analogies to the last-mentioned ones by confronting side by side *in situ* layers of heterogeneous tissues, which in normal development form distinct constituents of one anatomical region, namely the eye region. Pieces of tissues of various extent were mechanically removed from the eye-forming region of chick embryos of from 2 to 30 somites, and more or less wide gaps created. The organogenetic movements of the intact neighbouring regions reduce the gap rapidly and progressively, so that the fringes of severed tissue layers—which in normal development never establish a material continuity with each other—may be brought into direct contact side by side in the same plane. In this way, presumptive epidermis and retinal tapetum, nervous or pigmented layer of the retina and epithelium of the lens vesicle, &c., can be confronted.

The problem was to test whether in the new conditions the heterogeneous laminae could fuse with each other or whether they were unable to make connexions; and further, whether any changes in the normal course of the histological differentiation of the cells of one of the tissues might take place under influences spreading from the neighbouring cells of the other tissue in the fusion area.

The results can be briefly summarized as follows: the mesenchyme never establishes side-by-side connexions with epithelial layers (epidermis, epithelial wall of the brain, conjunctival epithelium, &c.). At first the mesenchyme covers the gap existing between the free edges of the severed epithelial lamina. Soon, however, the epithelial cells of the borders flatten and slide over the mesenchyme. In this way the cut edges of the epithelial lamina make contact with each other and fuse.

A transitory side-by-side fusion of epidermis and the wall of the anlagen of the brain occurs fairly often; however, each tissue retains its proper differentiation

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capacities and in later development the epidermis tends to separate from the wall of the brain; mesenchyme slides in between epidermis and brain and proliferates.

On the contrary, the cut borders of presumptive retina and lens epithelium, of tapetum and epidermis, of tapetum and conjunctival epithelium, form lasting and firm side-by-side connexions with each other. In such instances the affinity between the elements of the confronted tissues appears to be strongly positive. However, each of the tissues which at the time of the operation had not yet undergone any apparent differentiation retains through later development its own capacities for differentiation in regard to shape and arrangement of cells, and internal structure of its cellular components. Groups of cells showing transitional morphological characters between the two confronted tissues are never to be found along the fusion line. These facts seem to suggest that no special influence is exerted from one tissue on the other through the narrow area of contact.

Spreading of substances which can determine the fate of cells or change to some extent the course of their differentiation seems to occur in normal development, e.g. in supporting tissues. Many authors in fact maintain that striking examples of infectious propagation of differentiation are to be found in cartilage and bone tissues.

The differentiation of osteoblasts from the perichondral cells when the laying down of the perichondral bone ring of the cartilaginous diaphysis starts has been viewed as the consequence of the spreading from cartilage of an inductive substance (*osteogenin*, Lacroix). Such a substance determines the progressive differentiation of fibroblasts (assimilatory induction). Evidence was produced that ethanol extracts of bone or marrow (Levander) and of epiphysial plate (Lacroix) can induce the differentiation of fibroblasts of the loose or fibrous connective tissue into chondroblasts or osteoblasts.

An analogous interpretation has been advanced for the results of the following experiment: rib cartilage (and even the elastic cartilage of the ear of the rabbit) freed from its enveloping perichondrium and grafted to the epiphysial plate of the tibia undergoes structural changes similar to those of the epiphysial disk itself, viz. serial alignment and hypertrophy of cells (Lacroix). These last processes never occur in normal rib or ear cartilage *in situ* and represent preparatory steps for the replacement of cartilage with endochondral bone.

Pending experimental demonstration, it might be assumed that also the formation of *secondary* bone tissue, which is laid down during bone reconstruction, depends on the spreading of substances from the neighbouring bone tissue. These substances might determine the differentiation of fibroblasts, enclosed in the network of the vascular channels of bone, into osteoblasts. Fibroblasts are apparently at rest in the vascular channels where renewal of bone tissue does not occur; but, as soon as bone resorption sets in, the newly formed cavities are filled with fibroblasts and blood-vessels. The fibroblasts which line the wall of resorption cavities gradually become converted into osteoblasts which lay down concentrically arranged layers of bone matrix. This process suggests the existence of some inductive agent which diffuses from the freshly resorbed wall of the vascular channels.

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The Effect of Excess Vitamin A on the Differentiation of Chick Ectoderm in Culture[1]

by HONOR B. FELL, F.R.S.[2]

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THIS investigation was made in collaboration with Sir Edward Mellanby, F.R.S. (National Institute for Medical Research, Mill Hill). It has long been known that in animals suffering from vitamin A deficiency, mucous epithelia are replaced by keratinizing epithelium of the epidermoid type. Since keratinization is promoted by lack of vitamin A, it seemed possible that it might be inhibited by an excess of the vitamin. Series of cultures were therefore made in which explants of the ectoderm and underlying tissue from the trunk and limbs of 6–7-day chick embryos were grown by the watchglass or hanging-drop methods; the explants from one side of each embryo were grown in normal medium, and corresponding fragments from the opposite side in medium to which about 1,800 i.u. per 100 c.c. of vitamin A had been added (+A medium).

After 7 days' cultivation the controls in normal medium had formed a keratinizing, epidermoid epithelium, but keratinization was completely suppressed in the explants grown in +A medium. After more prolonged cultivation a remarkable metaplasia occurred in the treated cultures, the epithelium of which became transformed into a mucus-secreting, often ciliated epithelium similar to that of the normal nose.

This change was reversible. When the explants were transferred from +A to normal medium, the secretory tissue already formed showed a sudden spurt in development, but the basal layer of the epithelium produced no new secretory cells. Instead the basal elements proliferated to form a squamous epithelium, which eventually keratinized, beneath the mucous layer. The mucous cells, after their initial outburst of activity, degenerated and were shed. The histogenesis of the reversal of the mucous metaplasia induced in the ectoderm by restoring the normal concentration of vitamin A was closely similar to that of the squamous metaplasia induced by vitamin A-deficiency in, for example, the nasal mucosa of the intact animal (cf. Wolbach & Howe (1925), *J. exp. Med.* **42,** 753).

It is uncertain whether the vitamin A was directly responsible for the mucous

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metaplasia, or whether it merely inhibited keratinization. In the latter case it must be assumed that the ectoderm of the trunk and limbs, being blocked from continuing its natural course of differentiation, pursued an alternative path which normally it follows only in the nasal region.

Humoral and Tissue Factors in Tissue Differentiation

by P. J. GAILLARD[1]

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It was shown by Gaillard (1931, 1935, 1942) that pure strains of fibroblastic cells are very sensitive to those differences in the culture media which go hand in hand with the process of ageing of the donors used for preparing the media, the dimensional growth of the explants being used as a criterion. Moreover, strains of different origin reacted in a different way, thus demonstrating that cells which are morphologically alike can behave quite differently *in vitro.* When cultivating such a pure strain derived from the anlage of the frontal bone of a 15-day-old chicken embryo successively in a range of culture media prepared from 10-, 15-, and 18-day-old embryos, serum from a newly hatched chicken, and serum from an adult hen, histological bone formation occurred after 12 days of cultivation. This means that the realization of this obviously existing potentiality depended on the composition of the successive media.

The same principle emerged from experiments with the cartilaginous primordium of long bones from rat embryos (Gaillard, 1942; Verdam, 1946) and from human and chick fetuses (Zaaijer, 1953). During the cultivation of thyroid gland tissue derived from 8-day-old chicken embryos (Gaillard, 1949*a*, 1952) the 'age' of the medium also appeared to be decisive for the development of organotypical structure.

These and other observations led to a chemical analysis of the culture media used, but so far no conclusive results have been obtained.

Another factor came under consideration through cultivating human parathyroid tissue (Kooreman & Gaillard, 1950*c*), human ovarian cortex (Gaillard, 1949*b*, 1950*a*, 1950*b*, 1951), and rabbit anterior hypophysis tissue (Gaillard, 1942). In these cases only those cultures tended to show an organotypical character which became completely encapsulated by a layer of epithelial cells some days after explantation. Thus in cultures of ovarian cortical tissue a complete degeneration of the parenchyma even occurred when no capsule of germinal epithelium was present. Likewise in the above-mentioned experiments of Miss Zaaijer with human bone primordia the encapsulation by a sheath of epidermal

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cells appeared necessary for a balanced development of the epiphysial versus diaphysial zones.

The role of the epithelial encapsulation, however, has not yet been fully analysed, although it seems highly improbable that mechanical factors are of great importance. In the case of the ovarian cortex it became evident that the epithelial cell layer gave rise to cord-like proliferations with numerous oöcytes and that this layer was thus responsible for regenerating ovarian cortical tissue, while the interior parenchyma completely degenerated. In the other cases, however, nothing of this kind could be found, but it became clear that some kind of mutual influence existed between epithelium and subcutaneous connective tissue and cartilage in Miss Zaaijer's experiments.

The importance of these tissue interactions as determining factors in histological differentiation processes was most clearly demonstrated in experiments with confronted explants of the anterior and posterior lobes of the rabbit hypophysis (Gaillard, 1937, 1942). In contrast with a series of other tissues, the posterior lobe tissue caused a typical change of structure in the adjacent parts of the anterior lobe fragments. By the 'disappearance' of acidophil cells and by the development of cyst-like structures some structural features characteristic of intermediate lobe tissue occurred, and indeed it was suggested that perhaps *in vivo* the continuous interaction of posterior and anterior lobe tissue is responsible for the development and existence of the intermediate lobe of the hypophysis.

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Interference of Amino-acid Analogues with Normal Embryonic Development[1]

by HEINZ HERRMANN[2]

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THE study of the reactions of the embryonic organism to chemical substances is one of the main approaches to the analysis of embryonic development. Well known is the use of the lithium ion which produces in embryos of diverse species a variety of developmental abnormalities. However, in the case of a substance like lithium an analysis of the particular sequence of chemical reactions which leads eventually to the manifestation of a morphological abnormality encounters a peculiar difficulty. Since lithium reacts with numerous proteins, a wide range of reactions has to be considered as the initial step in the teratogenic mechanism.

For this reason it seemed advantageous to consider substances as potential teratogenic agents which are known to react only with one or a few definite chemical components of the cell. A class of substances which seems to fulfil these requirements is known in biological chemistry as 'analogues' (Woolley, 1950, 1952; Martin, 1951). Because of their close resemblance in chemical structure to vitamins, metabolic intermediaries, or other cell constituents these analogues interfere specifically with the proper function of the corresponding natural substances. Thus at least the starting-point of the disturbed mechanism is indicated and its further analysis greatly facilitated. Malformations in the chick embryo have been produced with a series of vitamin analogues. This work has been recently summarized by Cravens (1952). From previous studies by other authors very little has become known about the role of a disturbed protein metabolism in abnormal morphogenesis. Since amino-acid analogues offer the possibility of a more direct interference with protein metabolism, the use of such analogues seemed of interest in this connexion. The following outline is a preliminary report concerning disturbances in the development of chick embryos which were brought about by some amino-acid analogues.

In the present series of experiments tests were carried out on explanted chick embryos. The *in vitro* technique provides simplified conditions under which the developing embryo can be subjected to a variety of experimental factors and the

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results can be more readily observed and analysed.[1] Chick embryos were removed from the egg after about 24 hours of incubation. In most experiments embryos from the late headfold stage up to the 6-somite stage were selected. Controls and experimental embryos of exactly matching developmental stages were used for comparison. The embryos were transferred to the surface of a mixture of agar and an extract of egg. A total volume of 2 ml. of this nutrient was used. Except for small modifications the procedure for explantation and maintenance follows a technique described by Spratt (1947). The explanted embryos were kept in the incubator for about 18 hours. During this period the control embryos develop the primitive organs which correspond to those of a 15–20-somite embryo developing in the egg; the primitive central nervous system, optic vesicle and otocyst, a beating heart, a regular string of somites, &c., can be observed. Thus, while the period of explantation is short, it includes very fundamental steps in organogenesis.

In the present experiments two groups of analogues were used. The one comprised analogues of the aromatic amino-acid phenylalanine, the other group included analogues of the aliphatic amino-acids leucine and valine.[2] The structural formulae of the analogues and of the corresponding natural amino-acids are found in Table I. With the analogues of phenylalanine, i.e. thienylalanine, and *p*-chlorophenylalanine, abnormal forms of development were obtained at concentrations of the analogue in the culture medium of 2·5 and 1·5 mg./ml. respectively. Ortho-chlorophenylalanine was without any effect at a concentration of 1·5 mg./ml. One of the main defects which was observed under these conditions was a retardation of growth. As an example, data for four controls explanted at the headfold stage gave an average length of 4·0 mm. at the end of the incubation period, while five embryos developing on a thienylalanine medium reached only a length of 2·9 mm. If the natural amino-acid phenylalanine and the analogue were added to the medium simultaneously and in equal concentrations, the inhibition of growth was reversed at least in part (5 embryos with an average length of 3·4 mm.). In addition to the inhibition of growth, a variety of abnormal forms of development were observed. Very prominent were a zigzag neural tube, an irregularly formed brain, and the somites were often smaller and irregular in shape. However, the addition of phenylalanine did not reverse the abnormal forms of development.

The analogues of leucine and valine[3] interfere with normal development at much lower concentrations than the analogues of phenylalanine. Effects of the chlorine derivatives were observed regularly with 0·25 mg./ml. and of bromoallylglycine with 0·15 mg./ml. At these concentrations the inhibition of growth was slightly less than the inhibition found with thienylalanine. However, the leucine analogues seem to affect mainly the increase in length and less the increase in the other dimensions, while thienylalanine affects more evenly the growth in all dimensions.

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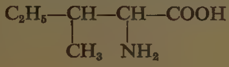
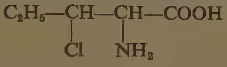
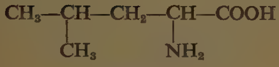
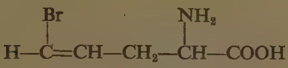
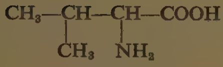
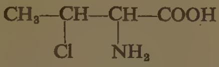
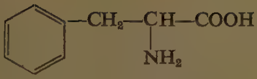
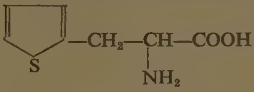
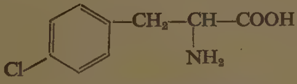
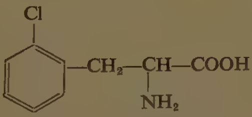
[1] The work using this technique was carried out at our laboratory by U. Rothfels and M. Curry.

[2] We are indebted for the supply of chlorine analogues of phenylalanine, leucine, and valine to Dr. H. J. Klooster (U. Colo.), and for the bromoallylglycine to Dr. K. Dittmer (U. Florida).

[3] In the remaining part of the paper reference is made only to results obtained with leucine analogues. Parallel experiments with the corresponding chlorine analogues of valine gave essentially the same results.

TABLE 1

Natural amino acids and their analogues used in experiments

Natural amino acids	*Analogues of isoleucine, leucine, and valine*
 $C_2H_5{-}\underset{CH_3}{\underset{\vert}{CH}}{-}\underset{NH_2}{\underset{\vert}{CH}}{-}COOH$ *isoleucine*	 $C_2H_5{-}\underset{Cl}{\underset{\vert}{CH}}{-}\underset{NH_2}{\underset{\vert}{CH}}{-}COOH$ α-NH$_2$-β-*chloro-valeric acid*
 $CH_3{-}\underset{CH_3}{\underset{\vert}{CH}}{-}CH_2{-}\underset{NH_2}{\underset{\vert}{CH}}{-}COOH$ *leucine*	 $H{-}\overset{Br}{\overset{\vert}{C}}{=}CH{-}CH_2{-}\overset{NH_2}{\overset{\vert}{CH}}{-}COOH$ DL-ω-*bromoallylglycine*
 $CH_3{-}\underset{CH_3}{\underset{\vert}{CH}}{-}\underset{NH_2}{\underset{\vert}{CH}}{-}COOH$ *valine*	 $CH_3{-}\underset{Cl}{\underset{\vert}{CH}}{-}\underset{NH_2}{\underset{\vert}{CH}}{-}COOH$ α-NH$_2$-β-*chloro-butyric acid*
	Analogues of phenylalanine
 *phenylalanine*	 β-*2-thienylalanine*
	 *para-chlorophenylalanine*
	 *ortho-chlorophenylalanine*

The most characteristic abnormality obtained with the leucine analogues consists in an impairment of the segmentation of somites, that is, in a failure of the somites to separate in the direction of the longitudinal axis of the embryo. The chlorine analogues exert this effect only during a short time. Two to four somites which normally separate 3–5 hours after explantation remain fused and thus form what we call a 'somite block'. Somites which form later separate again normally. In recent experiments we obtained evidence that the transient nature of this effect is due to the lability of the chlorinated analogues. In corroboration of these experiments we found that with a stable analogue like bromoallylglycine the failure of the somites to separate is much more extensive and usually involves all somites which enter the state of separation after the analogue is added.

The effects observed with the chlorine analogues of leucine are irreversible on addition of leucine. This irreversibility may also be a consequence of the labile nature of the chlorine analogues. During their breakdown these compounds may form covalent bonds with the tissue proteins which are not affected by the addition of the natural amino-acid. Such a possibility is borne out again by the use of the stable analogue bromoallylglycine, the effects of which are reversible. Whether there exists a dependence of the reversibility of the effects of bromoallylglycine and thienylalanine upon the stage of development of the embryos is now being investigated.

Histologically the process of the separation of somites consists in the rearrangement of the cells in the mesodermal plate and the formation of mesenchymal tissue which forms fibrous septa between the somites. Which phase of this process is actually suppressed is now being investigated. In considering the morphological mechanism of the separation the question arises whether the decrease of the growth rate of the embryo by leucine analogues may prevent the somites from separating. From our experimental material this possibility seems unlikely. We found equally extensive blocks of somites in embryos in which no retardation of longitudinal growth was observed after addition of leucine analogues, and the retardation of growth by thienylalanine leads only in very rare instances to the formation of 'somite blocks'.

So far we have found no evidence indicating that the suppression of the separation affects the differentiation of the somite into a dermomyotome and a sclerotome. In sections the development of these tissue components is found to have progressed to about the same extent in 'somite blocks' as in normal somites.

The chlorinated leucine analogues very rarely cause disturbances of the central nervous system. There may be a somewhat higher incidence with bromoallylglycine, but a greater number of experiments is required to make these figures more definite.

Summarizing the description of our results the striking differences in the effects brought about by the two groups of analogues may be pointed out. Phenylalanine analogues produce malformations only in concentrations 10 times higher than those found to give malformations with leucine analogues. Phenylalanine analogues suppress somite separation only as a rare exception, while this anomaly can be found regularly with leucine analogues. On the other hand, abnormally small and irregularly shaped somites are characteristic for thienylalanine and appear only rarely with leucine analogues. Abnormalities of the central nervous system are brought about very frequently by phenylalanine analogues but very rarely by the chlorine analogues of leucine.

An attempt at a more specific interpretation of the observed effects seems premature. Even the more extensive studies of the effects of amino-acid analogues on the growth of bacteria and other lower organisms have, apparently, not yet led to more definite suggestions about the mechanism of their action (Dittmer, 1950). However, it is likely that in many instances the analogues interfere more or less directly with the formation of new proteins. An inhibition of the oxidation of the amino-acids, leading to a diminished energy production, and hence to a reduced synthesis of proteins, seems unlikely in view of the recent results of Frieden, Hsu, and Dittmer (1951).

For the problem of abnormal development there remains the question as to how a diminished rate of protein synthesis would give rise to such malformations as were observed in our experiments. Since the tested analogues are antagonists of amino-acids which occur in practically all cytoplasmic proteins, the impairment of the utilization of these amino-acids should give rise to suppression of protein formation in general. In this case different analogues should bring about, at least qualitatively, the same type of malformation. Since the results of our experiments show qualitatively different effects of different analogues, this assumption cannot hold. In this case we have to postulate that different analogues inhibit the formation of different proteins to a different extent. This postulate can be tested experimentally by the measurement of the rate of incorporation of labelled amino-acids into different proteins in the absence and presence of amino-acid analogues. If different analogues inhibit the synthesis of proteins to a different extent, the rate of incorporation of labelled amino-acids into different proteins should correspondingly be reduced by different degrees. Experiments to test this hypothesis are in progress in our laboratory.

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Basic Mechanisms in the Differentiation of Pigment Cells

by B. H. WILLIER[1]

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SOME of the basic mechanisms of cellular differentiation have been elucidated by an experimental analysis of the underlying factors and conditions which control the processes of differentiation of the pigment cell in the chick embryo. The pigment cell, owing to its singular capacity to synthesize melanin pigment granules, is an ideal cell for locating its site of origin and tracing its subsequent pathways to the skin and derivatives, the feather papillae. It has the further advantage that its specific behaviour is recorded as colour or colour pattern in the mature feather. In other words, by using the pigment-forming cell as a 'marker' many of its properties in time and space may be ascertained from the moment that it is first set aside as a specialized strain of cell until it reaches its ultimate position and visible expression in the growing feather papilla.

1. In the chick the pigment-forming cell is a specialized strain of cell which has been traced back to early phases of development, i.e. ultimately and exclusively to the neural crest. Just when and by what means it acquires the basic properties and mechanisms which distinguish it from other cell types is not known. The evidence, however, indicates that the pigment-forming cells are intrinsically different from the beginning of the experimental test. At least some of the neural crest cells already have or soon acquire the latent capacity to synthesize melanin pigment granules, from which time they are appropriately designated as melanoblasts. Sooner or later these cells when associated with a suitable tissue environment begin the synthesis of melanin granules, from which time they are designated melanophores.

Once endowed with properties peculiar to their kind, i.e. the combined capacity for migration and the synthesis of melanin pigment granules, the melanoblasts migrate from one position to another in a dorso-ventral direction apparently in strict conformity with the specific properties of the tissue substratum prevailing at the time along their pathways. In other words, the melanoblasts appear to be 'guided' by the specific and oriented conditions prevailing in the dermal tissue immediately subjacent to the skin ectoderm and in certain other tissues as well. In this manner the melanoblasts within the skin migrate to

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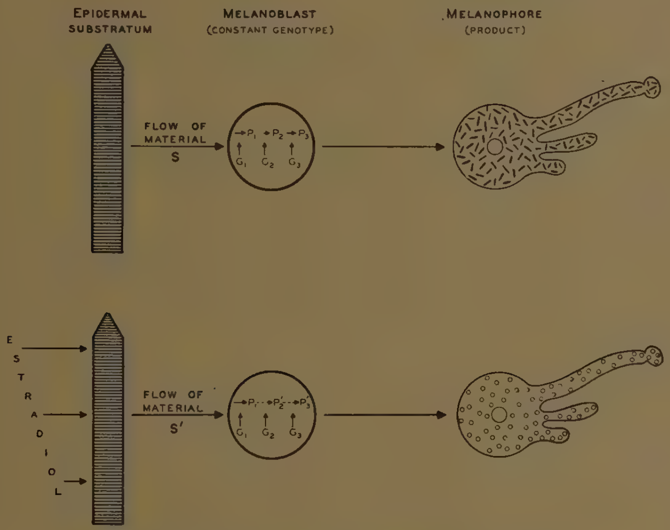
each of the developing feather papillae, at or near the base of which they become permanently established, thereafter constituting a constant source of melanoblasts to the feather throughout the life of the bird (Willier, 1948).

While in the relatively undifferentiated state, the melanoblasts have the capacity for growth (often increasing markedly in number), apparently by synthesizing more of the basic protoplasm capable of melanin synthesis (ordinarily melanin granules are not formed until after growth ceases). The melanoblasts may, therefore, be regarded in a sense as self-reproducing systems capable of growing indefinitely. The number of melanoblasts at a given stage and locus, however, is controlled not by a self-limitation of capacity for multiplication but by the tissue community in which they reside (Willier, 1948).

By virtue of specialized properties acquired during the earlier phases of differentiation the melanoblasts can combine, interact, and arrange themselves in certain specific and orderly ways relative to the developing epidermal tissue elements of the feather papilla. As an orderly morphogenetic system the feather papilla governs in sequence (1) the time of invasion of the melanoblasts, (2) their rate of multiplication and spatial arrangement in the developing epidermal elements of the feather, and likewise (3) the exact time and locus of transformation of a melanoblast into a melanophore with accompanying synthesis of melanin pigment granules. Further, it seemingly regulates (4) the order and direction of dendritic outgrowths from the melanophore as well as (5) the time that melanin granules are discharged and accepted by the epidermal cells. All processes of interaction between melanoblasts and the epidermal components are closely correlated in time (Willier, 1942, 1952).

2. Although the developing feather papilla clearly acts upon the melanoblasts in guiding their orderly behaviour and response, the specific kind of colour reaction given by the melanoblasts is basically dependent upon their specific genetic constitution. Invariably the kind of colour response is determined by the specific assemblage of colour or pattern-producing genes within them. In this respect the melanoblasts of the various breeds of fowl exhibit a wide diversity ranging from a lethal type (foreshortened life span resulting in white plumage) to a type capable of producing an intricate, sexually dimorphic colour pattern in the feather. Furthermore, melanoblasts of the colour-pattern genotype exhibit differences among themselves in reaction to sex hormones, some being sensitive whereas others are insensitive. The reaction of the hormone-sensitive melanoblast is looked upon as a matter of adjustment to the changes brought about in the feather papilla by the action of sex hormones. It is the harmonious organization of the feather papilla that integrates and utilizes sex hormones in directing the course of differentiation of the hormone-sensitive melanoblasts. The effect of the hormonal stimulus is indirect; it is mediated through the epidermal system of the feather papilla (Willier, 1950).

Finally, it is to be noted that all melanoblasts, irrespective of their genetic constitution, have in common certain basic properties such as the capacity to respond in an orderly manner to organized tissue environment and for the synthesis of melanin from precursor substances. So far as the underlying biochemical processes are concerned, little information is available save that tyrosinase or allied enzymes are in all probability localized in the melanoblasts. However, with respect to the terminal products in the differentiation process, the specific colour-producing genes have a decisive role in determining the range of reaction of which the melanoblast is capable. The range may be limited (self colour) or manifold (colour pattern). In the case of the hormone-sensitive melanoblast of the colour-pattern genotype, a change in the synthetic processes takes place in response to the action of the sex hormone. The sex hormones somehow affect one or more steps in the chain of biochemical processes involved in the synthesis of melanin, such that melanin granules which differ in morphology (size and shape), in colour, and presumably in chemical composition are produced (see Fig.).


EPIDERMAL SUBSTRATUM
MELANOBLAST (CONSTANT GENOTYPE)
MELANOPHORE (PRODUCT)
FLOW OF MATERIAL
S
$\rightarrow P_1 \rightarrow P_2 \rightarrow P_3$
G_1 G_2 G_3
ESTRADIOL
FLOW OF MATERIAL
S′
$\rightarrow P_1' \rightarrow P_2' \rightarrow P_3'$
G_1 G_2 G_3

HORMONAL MODIFICATION OF GENE-CONTROLLED PROCESSES IN MELANOBLAST DIFFERENTIATION

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Citations to the original literature and the detailed evidence on which this article is based are given in the review papers listed below.

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The Differentiation of the Crystalline Lens

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THE first sign of lens development in the vertebrate embryo is the appearance of a thickening of the head ectoderm in the area of contact between the ectoderm and the eye vesicle (lens placode).

The cytoplasm of the placode cells in the chicken embryo loses its vacuolization, the cells acquire a cylindrical form, the nuclei arrange themselves perpendicularly to the contact surface and move to the base of the cells (McKeehan, 1951).

At about the same time chemical changes take place in the placode cells. Ten Cate and Van Doorenmaalen (1950), using serological methods, were able to demonstrate the presence of specific lens proteins in the placodal stage of the lens in axolotl and chicken embryos. They could not entirely exclude the possibility of the presence of these proteins in the ectoderm at earlier stages, but with their sensitive method they got no indication of them before the placodal stage.

At present it is, therefore, impossible to decide whether the appearance of this early protein specificity must be regarded as a pre-morphological cell differentiation or whether it occurs synchronously with or after the morphological changes described by McKeehan.

One is inclined, however, to assume that processes of chemodifferentiation by which the typical building-blocks of the cells are produced will precede visible morphological changes.

It is generally accepted that the changes in the head ectoderm are due to an influence which the eye vesicle exercises upon that ectoderm at the contact surface (embryonic induction). One might consequently assume that the production of specific lens proteins takes place under the influence of an inductive action of the eye vesicle. To investigate this assumption the following experiments were done.

Saline extracts of presumptive lens ectoderm of axolotl neurulae (before the appearance of a lens placode) were mixed with rabbit anti-serum against lens proteins. No precipitin reaction could be demonstrated. Neither could a precipitin reaction be found when saline extracts of young eye vesicles (freed from head

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ectoderm) were mixed with the anti-serum. When, however, ectoderm extract and eye vesicle extract were mixed and incubated at 37° C. for 24 hours the mixture, when tested with the anti-serum, showed a precipitin reaction whereas the separate components of the mixture did not contain antigens after the same incubation (Woerdeman, 1950). Thus it seems permissible to assume that the production of specific lens proteins must be ascribed to an interaction between head ectoderm and the eye vesicle.

It is, of course, probable that this protein production takes place in the ectodermal cells normally as a very slow reaction and is only accelerated by substances passing from the eye vesicle into the ectoderm, but it is also imaginable that it would not occur at all without the influence of the eye vesicle and that enzymes of the cytoplasm of the ectodermal cells have to be activated or co-enzymes to be delivered by the eye vesicle. Substances may also pass into the ectoderm which together with other ectodermal substances may be combined to form lens proteins. Still another possibility is that lens proteins may be present in the ectoderm in a masked form and be unmasked. It seems anyhow to be proved that their demonstrability in the lens placode of amphibians is a result of an action of the eye vesicle on the surface ectoderm.

The objection can be raised that in some amphibians (e.g. *Rana esculenta*, Spemann, 1912; *Xenopus laevis*, Balinsky, 1951) the lens can apparently develop independently when the eye vesicle is absent. In *Rana esculenta*, however, it is very probable that lens induction occurs at a very early stage (open neural plate) and takes only a very short time (Woerdeman, 1952). The same may be the case in *Xenopus*. I see therefore no reason to admit that some amphibians must be excepted from the general rule that lens formation is produced by an induction process.

When lens placodes of young frog neurulae are extirpated and grafted under the ectoderm of the abdominal wall or when they are explanted into saline solutions, they develop into small undifferentiated vesicles. In some cases, however, some of their cells show a transformation into lens fibres, but this happens only when somewhat older donors are used (Woerdeman, 1941).

When undifferentiated lens vesicles are transplanted in the manner mentioned above, they develop into small lenses with an irregular mass of lens fibres. There are indications to show the presence of a very short period during which no difference in fibre-forming potency exists between the exterior and the interior wall of the lens vesicle, but very soon fibre formation originates only from the interior wall which has been in contact with the presumptive nervous layer of the retina.

These observations, together with many data from the literature on lens development, indicate that for a normal differentiation of the lens a lasting influence of the eye cup on the lens anlage is necessary.

Most of my observations mentioned above were made on larvae of *Rana esculenta*, and here again the objection may be raised that in Spemann's paper of 1912 well-differentiated lenses are to be seen which have developed independently of an eye cup.

In Spemann's laboratory the eggs were often kept at a low temperature before the operation. Ten Cate (1946) showed that under these circumstances the chemo-differentiation is less retarded than the morphological processes. Before the cooled embryos reach the stage of operation, induction may have taken place during a much longer time than under normal conditions, and chemo-differentiation may have proceeded almost normally. This may explain why, after extirpation of the eye anlage in cooled neurula stages, a well-differentiated lens develops, whereas it fails to develop if the same operation is carried out on neurulae kept at room temperature. If this explanation is valid *Rana esculenta* would develop a lens in accordance with what has been shown for other amphibians and no discordance would exist between the result of my experiments on *Rana esculenta* (at room temperature) and the results of Spemann.

Our conclusion must therefore be that the induction process in amphibians is a process of long duration. It takes only a short time to start the formation of specific lens proteins in the ectoderm, and the development of a placode (the first visible morphogenetic phenomenon). When the inductive action continues, the placode acquires the potency of vesicle formation and of separating itself from the surface ectoderm. In a following phase a change takes place in the interior wall which enables its cells to form fibres (development of a medio-lateral polarity).

It is still questionable if all these differentiation steps are caused by one inductive factor. It might be assumed that there is a whole series of factors, each of which is responsible for a certain phase of lens development. We must bear in mind that during lens development the chemodifferentiation and morphogenetic processes in the eye vesicle and eye cup also proceed, and that consequently their inductive influence may be changing continually. I need only mention the gradual disappearance of glycogen in the cells of that part of the eye vesicle which becomes invaginated (Woerdeman, 1933).

On the other hand, our experiments have shown that eye vesicles and eye cups of various phases of development transplanted under undifferentiated ectoderm will always induce the same sequence of morphogenetic processes, i.e. formation of a placode, transformation into a vesicle, separation of the vesicle from the surface ectoderm, and differentiation of one of its walls into lens fibres. When an old eye cup is transplanted under indifferent ectoderm, it never induces the direct production of lens fibres in the surface ectoderm.

The various steps of differentiation seem to be dependent one on the other, and this phenomenon indicates another possibility, namely, that the inducing agent remains unchanged but that its effect depends on the preceding changes which have occurred in the reacting ectoderm.

Various experiments have demonstrated that the eye cup preserves the capacity of inducing lenses for a long time, much longer than is needed for the normal lens induction (e.g. Woerdeman, 1939). Accepting the hypothesis of a continually changing inductive influence, we should have to admit that the various phases of induction overlap each other considerably. Accepting the hypothesis of the existence of one and the same inductive action of the eye cup throughout lens development, one has to consider this action as the stimulus which keeps going a certain fundamental process (e.g. formation of lens proteins) which is the indispensable source of modifications in the lens anlage leading to its final differentiation.

In some of the experiments cited above in which eye cups of various ages were transplanted under undifferentiated ectoderm, I also used material of old blastulae or very young gastrulae and made it react with old eye cups. I observed the development from this material of lens vesicles which showed a marked difference from normal lens vesicles. Whereas the cells of the latter do not possess vitelline platelets in their cytoplasm, the cells of the former contained a large quantity of vitelline platelets (Woerdeman, 1938). It seems possible, therefore, to induce lens formation in cells which have not yet passed through some of the processes of chemodifferentiation, although normally these processes are passed before lens formation starts. Since I killed my operated animals rather soon after the experiment I have not been able to observe the older stages of differentiation of these 'too young' lens vesicles. It might be interesting to repeat the experiments and to follow the fate of the induced lens vesicles (using also histochemical methods) to see if certain morphogenetic processes are dependent on or independent of preceding developmental processes in the cells.

The only conclusion which I wish to draw at present is that the competence for placode and vesicle formation is already established in the ectodermal cells at an early stage of development. They react, however, only after having made contact with the eye vesicle.

Another observation may be included here. When in frog embryos the lens vesicle which has separated from the surface ectoderm is removed through a small incision in the ectoderm, the wound closes and the regenerating ectoderm is able to form a new lens. This shows that shortly after lens formation the ectoderm has not yet lost its competence to be induced to lens development (Woerdeman, 1952).

Returning now to the further differentiation of the crystalline lens I must mention another influence of the eye cup on fibre-formation.

In the amphibian lens the growth of the fibres is directed in such a way that their ends meet in two lens sutures, an anterior one coinciding with the plane of the foetal fissure of the eye cup and a posterior one perpendicular to the anterior. By a series of experiments which will not be described in detail, I tried to investigate if there exists a causal connexion between the bilaterality of the eye vesicle (expressing itself in the formation of the foetal fissure) and the direction of growth of the lens fibres (resulting in the formation of two sutures, one of which coincides with the plane of the foetal fissure).

These experiments consisted in excision of the presumptive lens ectoderm, rotation through 90° and reimplantation, or in rotation of the eye vesicle or of the presumptive group of eye cells in the neural plate through 90° leaving the presumptive lens ectoderm in its place and normal orientation.

From these experiments it was concluded: (*a*) that the extent of the fibre-forming area of the interior wall of the lens vesicle depends on the area of contact with the eye vesicle, (*b*) that abnormally invaginated eye cups which have more than one area of contact with the lens vesicle induce more than one cone of fibres, and (*c*) that the direction of the growth of the lens fibres depends on the direction in which the eye vesicle invaginates (Woerdeman, 1932, 1934).

There must be assumed to be differences in various parts of the wall of the eye vesicle which later invaginates and which makes contact with the interior wall of the lens vesicle. These differences are manifested in the number of mitoses, content of glycogen and ribonucleic acid, &c. The pattern of these differences in the exterior wall of the eye vesicle seems to be responsible for the way in which the lens fibres start growing. I cannot exclude, however, that regulative forces acting within the lens vesicle also play a role in the establishment of the final structure.

Hitherto I have restricted my description to the influence which the eye vesicle and eye cup exercise on lens development. On the other hand, there is no doubt that the lens anlage influences the differentiation of the eye cup. I have only to mention the suppression of the lens-forming capacity of the border of the iris by the presence of a lens in some amphibia. But I will not enter into a description of this aspect of the interaction between eye cup and lens.

It was my intention to show by some examples that the crystalline lens is a most favourable object of study for one of the topics of our symposium: intercellular relations in differentiation.

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X-Ray Methods in Histochemistry

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THE aim of histo- and cytochemical studies is to identify and eventually to quantitate substances on a microscopic level. This necessitates isolating extremely small areas for analysis, preferably in the intact biological tissue. Physical methods have proven to be of value in this respect, in particular the radiant energy micro-absorption techniques (Fitzgerald & Engström, 1952).

The field of histochemistry is rapidly developing. In this summary, X-ray methods that have found use in histochemistry and histophysiology will be briefly discussed. Critical reviews (Glick, Engström, & Malmström, 1951; Fitzgerald & Engström, 1952) have recently been published.

A. FORMATION OF X-RAY IMAGES WITH HIGH RESOLVING POWER

The usual principle that has been adopted to obtain X-ray images of high definition is to place the object in close contact with a fine-grained photographic emulsion and make the exposure with X-rays as parallel as possible. The best emulsions, Eastman Kodak Spectroscopic Plate 548 or 649, Kodak Maximum Resolution Plate, Lippman emulsion (Gevaert), have a resolving power of about 1 μ. It is easy to arrange the geometry of the X-ray beam in such a way that the geometrical unsharpness (penumbra) is far below the resolving power of the emulsion. This is possible by having a relatively small focal spot in the X-ray tube, a thin sample in close contact with the photographic emulsion, and a relatively long distance between the focal spot and the sample-film. The method of microradiography described above can thus give a resolution of about 1μ and has been used to image a variety of tissues and cells by X-rays, for example, bone tissue (Amprino, 1952; Amprino & Engström, 1952), gastric mucosa cells (Engström & Malmström, 1952), nerve-fibre (Engström & Lüthy, 1950), giant salivary gland chromosomes (Engström & Ruch, 1951), &c.

The method of primary magnification introduced by von Ardenne (1940) has recently been further developed by Cosslett & Nixon (1951). This method depends upon making an enlarged shadow micrograph of the sample and necessitates a very fine focus. Intensity limitations make it doubtful if this method will give a resolving power much better than the simple microradiography, at least when it concerns biological objects which require very soft X-rays for contrast. The

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shadow X-ray microscope has recently been greatly improved by Cosslett and Nixon. The greatest hopes for an increased resolving power in the X-ray image formation are on the real X-ray microscope (Kirkpatrick *et al.*, 1948, 1950). Thus, using grazing incidence on polished, curved surfaces, a real enlarging system has been constructed. The resolving power of the X-ray microscope is expected to fall somewhere between that of the light and electron microscopes (1950).

B. DETERMINATION OF THE DRY WEIGHT OF HISTOLOGICAL AND CYTOLOGICAL STRUCTURES

A microradiogram registered with 8 to 12 Å X-rays can be evaluated in terms of the dry weight of the different structures in a dried smear or section of a soft biological tissue (Engström & Lindström, 1950). The procedure involves the photometric evaluation of the X-ray absorption in the various biological structures, a procedure which can cause difficulties. In most cases a reference system is printed on the fine-grained emulsion simultaneously with the microradiogram and with the same X-radiation. Improved methods have been described for manufacturing such small reference systems (Brattgård, 1952; Brattgård & Hallén, 1952; Brattgård & Hydén, 1952). As very small areas are measured photometrically it must be ascertained that a sufficient number of silver halide grains are within the measurement area in order to reduce the pure statistical error in the photographic process. The method of weighing biological structure will be greatly improved when the X-ray microscope (Kirkpatrick & Baez, 1948; Kirkpatrick, 1950) is available.

The accuracy of the cytochemical X-ray weighing procedure is not high compared to standards in analytical chemistry. There are several factors that contribute the relative low analytical accuracy (Fitzgerald & Engström, 1952; Glick, Engström, & Malmström, 1951). Most of these factors are inherent in the objects themselves. Factors like the inhomogeneous distribution of the absorbing material, artifacts introduced by fixing, sectioning, and drying the biological sample, errors in determining the length of the absorbing path, are all difficult to master (Glick, Engström, & Malmström, 1951). The methodological errors such as defects in the fine-grained photographic emulsions, systematic errors (generally not exceeding 5 per cent.) introduced by the varying composition of the biological material, and errors in photometry must also be carefully considered.

Despite the relative large errors (Lindström, 1953*a*, 1953*b*) in the weighing procedure there are several problems in histo- and cytochemistry to which the method of X-ray weighing can be applied.

C. HISTOCHEMICAL ELEMENTARY ANALYSIS BY ABSORPTION

When the amount of an element is determined by X-ray absorption measurements, the characteristic absorption discontinuities are used (Glocker & Frohnmayer, 1925). For histochemical elementary analysis the absorption of monochromatic X-rays is measured on either side of the absorption edge (Engström, 1946). The procedure thus involves the transmission measurements of at least two wavelengths. There is a required minimum surface density of the element to be analysed (Engström, 1946). Therefore it is not possible to make histochemical elementary analysis of thin sections by X-ray absorption analysis.

The X-ray absorption method for histochemical elementary analysis has the advantage that very small samples (the area of an ordinary mammalian cell) can be measured, but in that sample the elements must have a high concentration. For that reason the X-ray absorption histochemical analysis can at present only be applied to elements in relatively high concentrations (for example, carbon, oxygen, nitrogen, sulphur; calcium and phosphorus in bone tissue).

D. HISTOCHEMICAL ELEMENTARY ANALYSIS BY X-RAY FLUORESCENCE

Chemical analysis by X-ray fluorescence was early used in metallurgy and geochemical research (Hevesy, 1932; Hevesy & Alexander, 1933). In principle the sample is irradiated by X-rays of a suitable wavelength and the excited fluorescence radiation (secondary X-rays) is analysed spectroscopically. The method is suitable both for qualitative and quantitative analysis of elements with relative high atomic numbers (Hevesy, 1932). Recently there has been rapid technical development of the X-ray fluorescence methods, especially for industrial purposes.

The fact that the biological material consists of elements with low atomic numbers makes this method less applicable to studies in biology. However, it has been used in certain instances to determine, for example, zinc and iron. The structural resolution is poor.

The curved crystal image X-ray spectrograph introduced by von Hamos (1938, 1953) offers a possibility of analysing the fluorescence X-rays emitted from areas as small as 10μ by 10μ. The quantitative aspects of this method have been carefully considered (Hamos, 1953).

E. STEREOSCOPIC MICRO-ANGIOGRAPHY

Studying the function of the smallest blood-vessels is of considerable interest for the understanding of the function of organs and tissues. Barclay and his colleagues (1951) used a radiological technique to demonstrate the finest blood-vessels. They called the procedure micro-arteriography. This method has recently been critically examined (Bellman, 1952, 1953; Bellman & Engström, 1952). The radiological background for micro-angiography (Bellman & Engström, 1952) is simple straightforward microradiography, as described earlier in this survey. The great problem, however, is to fill all the fine blood-vessels with X-ray contrast. It can be said that the problem of micro-angiography is that of introducing the contrast (Bellman, 1952, 1953).

The application of stereoscopic microradiography (Berthold, 1941; Engström, 1951) to the problem of micro-angiography has added much to the value of the method (Bellman, 1953). A three-dimensional representation of the capillary net in an organ can now be obtained. Micro-angiography has been performed in living animals (Bellman, 1953), and by this technique it is possible to study the physiology of the finest blood-vessels.

F. MICRO X-RAY DIFFRACTION

The great importance of X-ray diffraction methods in studying biological systems is well illustrated by the results obtained from essentially homogeneous macroscopic samples. Information concerning molecular orientation, particle and molecular sizes, and in certain cases detailed molecular structure has been obtained.

Micro-diffraction (wide angle) has been used to study different problems of cellular organization (bone tissue by Amprino & Engström, 1952; starch grains by Kreger, 1946, &c.). Various types of microcamera have been designed (Jeffery, 1952) including such using a fine glass capillary as collimator. The use of very fine focus X-ray tubes (Ehrenberg & Spear, 1951; Ehrenberg & Franks, 1952) is of great importance in micro-diffraction, especially when studying long spacings (Ehrenberg & Franks, 1952) (low scattering angles). It can be expected that micro X-ray diffraction will find a wide use in histochemistry, especially for studying large spacings and for particle size determination from the low-angle continuous scatter (Guinier, 1952).

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Changes in the Differentiation Level of Transplanted Tumours

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IT has been well established that the serial transplantation of an originally highly differentiated mammalian neoplasm leads regularly to a gradual loss of most or all visible expressions of histological differentiation within the tumour tissue. In a tumour that has undergone many years of serial transplantation there can usually be detected no resemblance to the tissue of origin, but rather a similarity to early embryonic tissue. Simultaneously with these morphological changes a gradual increase in growth rate takes place. The completely anaplastic tumour with a long transplantation history grows usually at a very rapid rate as compared to the original spontaneous tumour. The growth rate of such tumours is, however, still inferior to, or, at its maximum, equal to the growth rate of embryos of the same species (Schrek, 1936; Klein & Révész, 1953), but has never been found to exceed it. These changes in growth rate and level of differentiation are usually coincident with a gradual decrease in the number of histocompatibility factors the presence of which in the implanted host is necessary for the progressive growth of the tumour. This decrease has been interpreted as somatic mutation (Strong, 1926), antigenic simplification (Gorer, 1948), or, more recently, as immunological selection from among the antigenic variants in a population of malignant cells, where the antigenic variants arise mainly as a consequence of disturbances in chromosome number (Hauschka, 1952).

The often correlated changes in growth rate, differentiation level, and antigenic strain specificity can be well studied in a series of tumours that arose in the same tissue of a certain inbred mouse strain at different times and have undergone different numbers of transfer generations. Considering, for instance, the readily available spontaneous mammary carcinomas of certain inbred mouse strains, it can be pointed out that the changes occurring during the prolonged transplantation of these tumours show many tendencies similar to those that have been observed during the period while the normal mammary gland of these high-cancer strain mice passes through the series of changes considered as precancerous and develops finally into the spontaneous tumour. These similarities may be only superficial, but it is intriguing to speculate that the gradual changes that can

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be observed while the spontaneous tumour is serially transplanted, and thus gets its life prolonged indefinitely, are merely a continuation of the gradual processes that have led from the normal tissue to the spontaneous tumour.

We have attempted to study several biochemical and growth characteristics within a series of tumours that have undergone varying numbers of transfer generations. One interesting quality that seems to be entirely absent on the higher levels of differentiation (and therewith correlated lower growth rate) is the capacity of the tumour cells to grow as free cells in the peritoneal fluid, forming there a concentrated suspension. This so-called ascites tumour form of growth has been found to be limited to tumours of the highest growth rate and anaplasticity, with a long transplantation history (Klein, 1951). A very great number of cell generations are necessary before cells appear and can be selected that are able to multiply in body fluids as dissociated free cells. Some results were obtained indicating that such cells may originate by mutations, using mutation in the broader sense, not necessarily meaning gene mutation (Klein, 1953). Such 'mutants' may have some significance with respect to the mechanism of metastasis.

Transplanted tumours that have originated at different times within the same tissue of the same inbred mouse strain are favourable objects for study of the changes in growth rate, differentiation level, and histocompatibility factor requirement that invariably follow serial propagation and the morphological or biochemical correlates of these changes. Such correlates may give information with regard to the apparently related changes that occurred earlier, during the cancerization of the normal cell.

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Determination of Mass of Nerve-cell Components

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THE method for determination of mass in biological material described by Engström & Lindström (1950) is founded on the fact that the absorption of X-radiation at 8–10 Å is proportional to the mass of the biological substance. In elaborating this method for quantitative analysis of nerve-cells and their components (Brattgård & Hydén, 1952) we found it important to consider the following sources of error:

1. The reference system.
2. The photographic process and the photometry.
3. The treatment of the material.

1. *The reference system.* The error in the reference system was investigated and a new method was elaborated at the Histological Department in Göteborg for the production of the thin cellulose foils which constitute the reference system (Brattgård & Hallén, 1952; Brattgård & Hydén, 1952; Hallén, 1953). We found, however, that this gravimetric method for the control of the mass per unit surface of the reference system involved too great an error. Therefore an interferometric method was worked out (Hallén & Ingelstam, 1952). This method determines the mass of the reference system with an accuracy of ±1 per cent.

2. *The photographic process and the photometry.* It was found that density measurements on a secondary magnification of the radiogram (the Lippmann-film) had a standard deviation of ±36 per cent. (Brattgård & Hydén, 1952, and unpublished), and this procedure had to be abandoned. Therefore we constructed a special microphotometer (Brattgård, 1952; Brattgård & Hydén, 1952; Hydén, 1952; Bourghardt *et al.*, 1953). The light intensity over a surface corresponding to 3–4μ^2 in the primary picture is measured by means of a multiplier tube and recorded automatically. The radiogram can be magnified 800 times. The construction and the results of the tests will be published elsewhere. The error of the film and the photometry was determined experimentally and found to be ±1·5 per cent.

3. *The treatment of the material.* It is recommended to use fresh, untreated material (Brattgård & Hydén, 1952; Hydén, 1952). The fixation and the additional treatment in the cytological procedure cause losses of mass in the cells (Table 1). If fixed material is used, microchemical determinations must be carried out simultaneously (Brattgård, 1952). Note that the fixation treatment dissolves

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relatively more substance from the nucleus of the nerve-cell than from the cytoplasm (see Table 1). This agrees well with the data obtained from other types of cells.

TABLE 1

Effect of fixation on the mass of the nerve cell.
Motor nerve-cells from adult rabbit

The thickness of the sections is comparable in both cases. The values are corrected and determined optically.

	Fresh frozen cells		*Carnoy fixed cells*
	Total mass 10^{-9} mg./μ^2	*After lipid extraction* 10^{-9} mg./μ^2	10^{-9} mg./μ^2
Cytoplasm . .	0·71±0·056	0·35±0·035	0·34±0·017
Nucleus . . .	0·48±0·016	0·30±0·037	0·13±0·008

The method used by us involves a chemical fractionation of the cell substance. The lipids are extracted by means of chloroform. By digestion with ribonuclease a reproducible fraction is obtained, which we studied chemically. For the time being we call this the 'nucleoprotein fraction'. For details see Brattgård (1952) and Brattgård & Hydén (1952).

Differentiation in nerve-cells. The effect of the removal of the periphery on the differentiation of motor neuroblasts in terms of the total amount of dry weight was studied. Neuroblasts and young nerve-cells of chick embryos were investigated. The somatopleur of the embryos had been removed at 60–70 hours' incubation. This material was kindly placed at our disposal by Dr. Karl Mottet, Dept. of Physiology, Yale University. The material was fixed in Carnoy's solution. The effect of the fixation procedure on the material was studied (Brattgård, 1952; Brattgård & Hydén, unpublished). Table 2 gives an example of the retardation in differentiation in terms of mass and also approximately per cell cytoplasm.

TABLE 2

Chick embryo

Total development: 6 days. Development after operation: 3 days.

	Control side	*Operated side*
	Young nerve-cells	*Corresponding cells*
Mass mg. × $10^{-9}/\mu^2$	0·65±0·050	0·35±0·040
Mass per cell cytoplasm mg. × 10^{-9}	123	10

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Influence of Certain Substances on the Fluidity of the Cytoplasm and the Fertilizability of the Egg of the Sea-urchin, *Psammechinus miliaris*

by ELSA WICKLUND, GEORG KRISZAT, *and* JOHN RUNNSTRÖM[1]

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AFTER nuclear maturation the eggs of *Psammechinus* (*Ps.*) *miliaris* are still 'underripe' with respect to their cytoplasm. This phenomenon is particularly conspicuous in *Ps. miliaris* from the Swedish west coast (see, for a summary, Runnström, 1949). In the small individuals of *Ps. miliaris* dredged from a depth of 30–40 metres this state of underripeness may last for months, so that full cytoplasmic maturity may be reached only in the latter half of August (Lindahl & Runnström, 1929). The littoral form of *Ps. miliaris*, on the other hand, has already attained cytoplasmic maturity in the latter half of June. Temperature conditions evidently play a decisive role in the phenomenon.

The swelling of the jelly coat surrounding the egg certainly plays an important role in the process of 'cytoplasmic' maturation. Removal of the jelly coat sometimes improves fertilization, but not always. This latter fact proves that the state of underripeness resides also in the cytoplasm itself.

A number of substances have been shown to remove the state of underripeness of the eggs. Among these substances proteins, like serum albumin, and several amino-acids were used (Runnström *et al.*, 1943; Runnström *et al.*, 1946; Wicklund & Gustafson, 1949). The same substances have an activating effect on the spermatozoa and prolong their fertilizing period (Wicklund, 1949; Tyler, 1950; Vasseur *et al.*, 1950; Tyler & Rothschild, 1951). The improvement is also brought about, however, when quite fresh sperm and underripe eggs are mixed in the presence of one of the substances mentioned. Prior to the addition of this the immature state of the egg cytoplasm is the limiting factor. When, on the other hand, fully mature eggs are mixed with aged sperm, the quality of these latter impairs fertilization. Addition of amino-acids or proteins now brings up the sperm to full activity and thus ensures normal fertilization. By appropriate experimentation it may prove possible to decide whether certain of the substances have a stronger action on the sperm than on the egg or vice versa.

The progress of cytoplasmic maturation can be followed in the eggs of *Ps. miliaris* with the aid of the 'hypertonicity test'. The eggs are brought into 2 ml.

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sea-water + 0·6 ml. 2·5 N NaCl. The underripe eggs are characterized by a prolonged state of wrinkling of the egg surface, while in the mature egg the wrinkled state is earlier replaced by a smooth state of the egg surface (Runnström & Monné, 1945; Runnström, 1949). This study has been taken up on a broader basis by Wicklund and Kriszat. The data reported below are taken from their experimental material, which still needs supplementing in many respects. They used three parallel methods to evaluate the state of fluidity or rigidity of the cytoplasm, viz. the hypertonicity test, the centrifugation of the eggs in sea-water made viscous by addition of gum arabic or dextran, or the centrifugation of the eggs

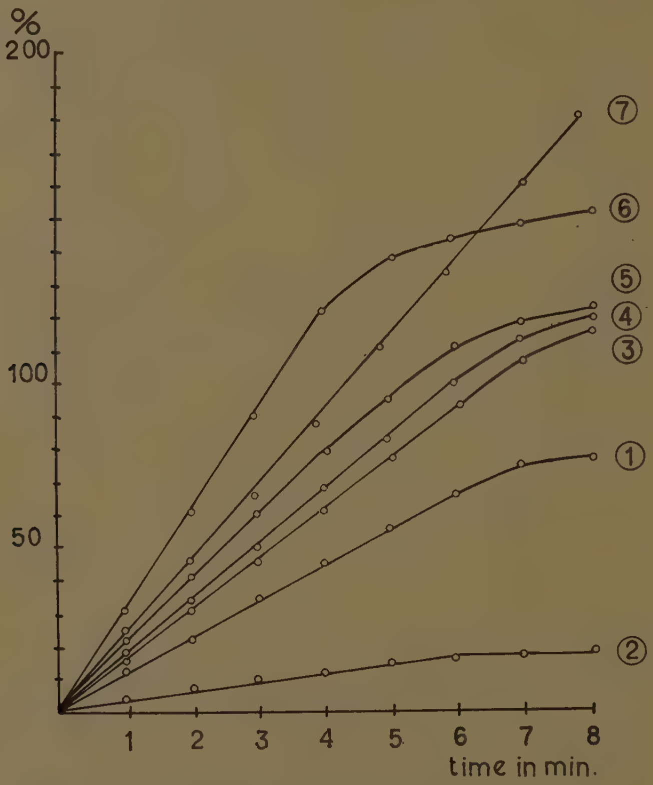

FIG. 1. The elongation of centrifuged eggs, expressed in percentages of the egg diameter, as a function of time. The eggs were suspended at the border between sea-water with different additions and an isotonic sucrose solution. The additions were the following: (1) none (control); (2) 'fucoidin' 0·05%; (3) glycine 0·05 M; (4) *dl*-alanine 0·05 M; (5) serum albumin 0·2%; (6) versene 10^{-4} M; (7) thioglycolate 10^{-3} M.

suspended at the border between sea-water and isotonic sucrose solution (according to E. B. Harvey, 1932).

The main result is that many amino-acids and proteins added to the sea-water increase the rate of smoothing of the eggs in a hypertonic medium. At the same time they decrease the inner rigidity of the eggs and also decrease the resistance against elongation of the suspended eggs. The last-mentioned change is the easiest to present in a graphical form; see Fig. 1.

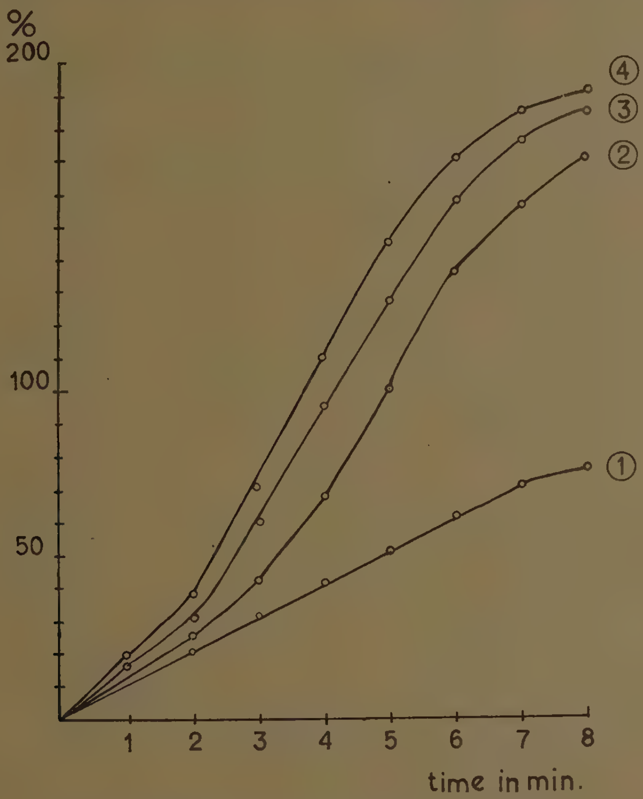

FIG. 2. See legend to Fig. 1. The additions to the sea-water were the following: (1) none (control); (2) a serum globulin fraction 0·2%; (3) human globin 0·2%; (4) silk peptone 0·2%.

The control eggs attain an asymptotic value after 7 minutes (acceleration 4,350 g.) with 75 per cent. elongation. An elongation of 110–120 per cent. is attained in presence of 0·05 M glycine, *dl*-alanine, and 0·2 per cent. serum albumin. Despite the more pronounced elongation there is an approach to equilibrium towards the end of the period of centrifugation. Fig. 2 shows the influence of two proteins, viz. human globin and a serum globulin fraction, and of silk peptone. They were used in a concentration of 0·2 per cent. It is found that these compounds have a very strong action on the elongation (170–190 per cent.). Some acceleration of the effect is found after about 2 minutes and at the end of the period of centrifugation the approach to an equilibrium state is again observed.

In Fig. 1 some curves do not pertain to amino-acids or proteins. Curve 6 refers to the effect of 10^{-4} M versene (ethylenediamine tetra-acetic acid). The maximum slope of the curve amounts to 30 per cent. per minute, while the corresponding value for the control is 10 per cent. per minute. The maximum slope for versene is almost twice that for 0·2 per cent. albumin and exactly twice that obtained in glycine, although the molar concentration of the latter substance is 500 times higher. Already after 4–5 minutes there is a turning-point on the curve. Thereafter the slope rapidly decreases.

The decrease in rate which occurs after a variable time of centrifugation might mainly reflect a resistance set by the rigidity of the interior cytoplasm.

The curve obtained for eggs subjected to 10^{-3} M thioglycolate in sea-water has a slope of 22–23 per cent. per minute, and this is constant for the duration of the experiment. This might mean that the internal resistance is still more decreased than in presence of versene.

The most remarkable result recorded in Fig. 1 is found in curve 2, which refers to an egg subjected to 0·05 per cent. 'fucoidin', a not fully pure preparation of this polysaccharide from the alga *Fucus vesiculosus*. The rate of elongation is here 1/5–1/4 of that in the control and about 1/12 of that in presence of 10^{-4} M versene.

Among other amino-acids *dl*-leucine caused an elongation at a rate of 1·9, methionine 1·6, and *l*-histidine 1·5 times the rate exhibited by the control, the amino-acids being tested in 0·05 M solutions. The values obtained for serine, oxyproline, and arginine were slightly higher than those of the control, but it has not been proved that this difference is significant.

On the whole, the amino-acids have proved to decrease the rigidity of the interior cytoplasm. The same holds true for versene. In particular, a comparison has been made between the elongation of the egg in the centrifugal field and the rate of smoothing of the egg surface in the hypertonicity test.

The typical course of events is that the eggs all remain wrinkled for a certain period; then the eggs begin to become smooth at a low rate, which rapidly increases until the percentage of smooth eggs approaches 100 per cent. after 30–40 minutes, depending on the material. This applies to the control. In presence of amino-acids the smoothing begins earlier. As a rule there seems to be a close correlation between the rate of elongation in the centrifugal field and the rapidity of smoothing of the surface. There are perhaps some exceptions to this rule, but the experimental basis must be enlarged before these exceptions should be considered.

It is quite obvious, however, that substances like silk peptone, globin, serum albumin, versene, or thioglycolate which cause a strong elongation also enhance the smoothing of the eggs very considerably. In 10^{-4} versene, for example, the eggs begin to become smooth after 2–3 minutes, whereas in the control all the eggs are still wrinkled 15 minutes after transfer to the hypertonic medium. Moreover, there is no membrane formation in the control, or at the most a thin irregular granular membrane appears. This disappears again when the eggs are brought back to the normal medium. In presence of proteins, however, a great number of the eggs present smooth, elevated membranes which when examined in the polarization microscope were found to have the retardation characteristic of a normal fertilization membrane. The elevation of a normal membrane occurred also in serum albumin and to a lesser extent in the amino-acids. The quantitative relations remain here to be elaborated.

It has been described above how the width of the jelly coat increases in connexion with the cytoplasmic maturation of the egg. The substances mentioned above all favour the swelling of the jelly coat. This was demonstrated for amino-acids, histidine, glycine, cysteine, tryptophane, and glutamic acid, and for diluted (1/10–1/15) rabbit serum by Runnström *et al.* (1946). The material was the littoral form of *Ps. miliaris*. The volume of the egg is $4{\cdot}9 \times 10^{-4}$ μl. Before swelling the jelly coat had a volume of $10{\cdot}2 \times 10^{-4}$ μl. In presence of 1/15 diluted serum the volume increased to 21×10^{-4} μl.

Borei & Björklund (1953) noted that versene caused a rapid swelling of the jelly of the egg of *Ps. miliaris* ('S-form'). The swelling process reaches its maximum after an exposure for 20–30 minutes. The average volume of the egg was $5{\cdot}3 \times 10^{-4}$ μl. The final volume of the jelly coat depended on the concentration of versene, as follows from Table 1.

TABLE 1

Concentration of versene	*Volume of the jelly coat* $\times 10^{-4}$
0	11·1 μl.
10^{-5} M	12·5 ,,
10^{-4} M	58·6 ,,
10^{-3} M	87·1 ,,

No change in volume of the egg itself could be demonstrated. On prolonged treatment (for more than 30 minutes) the diameter of the jelly coat gradually decreased. This is probably due to a beginning solvation of the jelly coat.

In presence of 0·05 per cent. 'fucoidin' the eggs remain wrinkled in the hypertonic solution for a longer period than eggs in pure hypertonic medium. Furthermore, the swelling of the jelly coat is much delayed in eggs exposed to 'fucoidin'. This is in keeping with the conspicuous difference that has been demonstrated to prevail between the control eggs and those subjected to 'fucoidin' with respect to the elongation in the centrifugal field.

Most significant in these experiments is the obvious parallel between the properties of the egg as reflected in the tests described above and the fertilizability of the egg. 'Fucoidin', 0·02–0·05 per cent., inhibits fertilization. In lower concentrations 'fucoidin' does not inhibit engulfment of the spermatozoon, but prevents membrane elevation (see Harding, 1951). Versene, proteins, and amino-acids, on the other hand, improve the membrane formation in eggs which show various degrees of refractoriness extending from complete failure of membrane formation to slight irregularities and decreased rate of membrane elevation, as compared with eggs in optimal conditions.

Finally it has been reported that addition of the substances in question brings about the formation of a normal 'activation membrane' in a hypertonic solution, while in this alone no membranes or very imperfect membranes appear. This last-mentioned result gives additional support to the conclusion that not only the sperm but also the egg is affected by the addition of the various substances under discussion. In many cases the effect may be on eggs and on sperm as well. It may be mentioned in passing that colchicine ($1{\cdot}5 \times 10^{-4}$ M) has a strongly elongating effect on the unfertilized eggs. In the initial period of centrifugation the rate of elongation was 3 times and increased after 3–4 minutes to a value 7 times greater than that prevailing in the eggs not treated with colchicine. Colchicine was also found to decrease the interior rigidity of the egg (cf. Beams & Evans, 1940). It was of interest that presence of or pretreatment with colchicine caused a considerable increase in the percentage of normal fertilization of underripe eggs, although the exposure to colchicine brings about disturbances already in the course of the first mitosis.

As to the interpretation of the facts described in this section, it is obvious that many of the substances improving the fertilizability and correlated properties of the egg are known for their metal-complex forming capacity. Borei & Björklund (1953) are, for example, inclined to interpret the action of versene as being due to a complex formation with heavy metals or with calcium and magnesium in the cytoplasm of the egg. Subsequent to removal of the metals a certain loosening of the intermolecular forces would take place. There is, however, one result that contradicts this conclusion, at least with respect to calcium. As mentioned above, the eggs become smooth after a short time in the hypertonicity test, if versene, 10^{-4} M, is present. If, on the other hand, the eggs are exposed to calcium-free medium, the eggs remain for a longer time in the wrinkled state than the control with normal calcium content (Runnström & Monné, 1945). One would thus expect that versene would prolong rather than abbreviate the smoothing of the eggs in the hypertonic medium.

Another efficient metal-complex former is 8-hydroxyquinoline. Only few observations were carried out with this substance. They definitely showed, however, that in 0·05 M concentration it causes a stretching of the eggs which considerably exceeds that observed in versene. This may be due to the difference in concentration. The molar concentration of the quinoline was in fact about twice that of calcium present in the sea-water. Nevertheless, the eggs became smooth very rapidly when immersed in the quinoline solution. They thus did not react as in calcium-free sea-water.

The substances which form metal-complexes certainly also have a strong tendency to form hydrogen bonds. As a consequence they are able to break up complexes of macromolecules which are kept together by means of hydrogen bonds. In this way a depolymerization takes place giving rise to a decrease in rigidity of the cytoplasm. Besides the breaking of hydrogen bonds, the complex formation with metals may play a role.

It has been reported recently (Hultin *et al.*, 1952) that sperm extract has a similar action on the eggs as that described above for certain proteins, versene, &c. The extract was prepared simply by washing the sperm with sea-water and then centrifuging down the spermatozoa. The supernatant obtained (S 1) increased the rate of elongation of the egg about twice. The smoothing of the egg surface in the hypertonic solution was also enhanced in presence of the extract. Extracts from the granular fraction of the egg (the 'membrane elevating factor of the egg', Hultin *et al.*, 1952) had a similar effect on the egg.

These briefly mentioned results with sperm and egg extract point also to the conclusion that the changes of the egg cytoplasm described above play an important role in the initiation of the development of the egg. From a different approach Runnström (1952) arrived at the conclusion that an increased fluidity and mobility of the cortical cytoplasm of the egg is necessary in the first steps following upon the attachment of the spermatozoon.

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